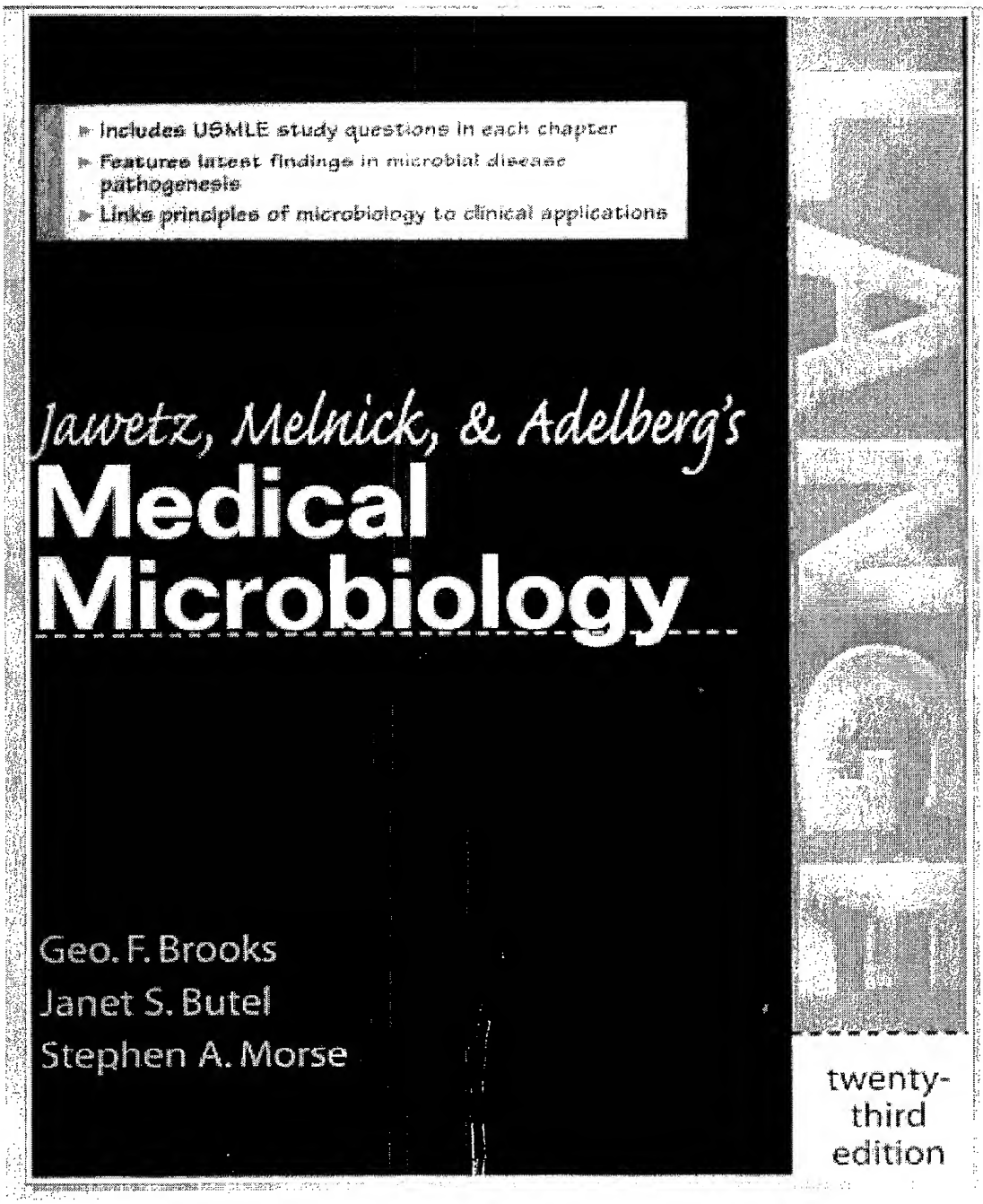
Exhibit A

Submitted as Exhibit to Amendment of January 28, 2009


Includes USMLE study questions in each chapter
Features latest findings in microbial disease pathogenesis
Links principles of microbiology to clinical applications
Jawetz, Melnick, & Adelberg's
Medical Microbiology
Geo. F. Brooks
Janet S. Butel
Stephen A. Morse
twenty-third edition

isms because they contain all of the enzymes required for their replication and possess the biologic equipment necessary for the production of metabolic energy. Thus, eukaryotes and prokaryotes stand distinguished from viruses, which depend upon host cells for these necessary functions.

VIRUSES

Viruses lack many of the attributes of cells, including the ability to replicate. Only when it infects a cell does a virus acquire the key attribute of a living system: reproduction. Viruses are known to infect all cells, including microbial cells. Host-virus interactions tend to be highly specific, and the biologic range of viruses mirrors the diversity of potential host cells. Further diversity of viruses is exhibited by their broad array of strategies for replication and survival.

A viral particle consists of a nucleic acid molecule, either DNA or RNA, enclosed in a protein coat, or capsid. Proteins—frequently glycoproteins—in the capsid determine the specificity of interaction of a virus with its host cell. The capsid protects the nucleic acid and facilitates attachment and penetration of the host cell by the virus. Inside the cell, viral nucleic acid redirects the host's enzymatic machinery to functions associated with replication of the virus. In some cases, genetic information from the virus can be incorporated as DNA into a host chromosome. In other instances, the viral genetic information can serve as a basis for cellular manufacture and release of copies of the virus. This process calls for replication of the viral nucleic acid and production of specific viral proteins. Maturation consists of assembling newly synthesized nucleic acid and protein subunits into mature viral particles which are then liberated into the extracellular environment. Some very small viruses require the assistance of another virus in the host cell for their duplication. The delta agent, also known as hepatitis D virus, is too small to code for even a single capsid protein and needs help from hepatitis B virus for transmission. Different viruses are known to infect a wide variety of specific plant and animal hosts as well as prokaryotes and at least one eukaryotic alga and one protozoan. Virus-like particles that seem to lack an infectious extracellular phase have been found in fungi as well as in several genera of algae.

A number of transmissible plant diseases are caused by viroids—small, single-stranded, covalently closed circular RNA molecules existing as highly base-paired rod-like structures; they do not possess capsids. They range in size from 246 to 375 nucleotides in length. The extracellular form of the viroid is naked RNA—there is no capsid of any kind. The RNA molecule contains no protein-encoding genes, and the viroid is therefore totally dependent on host functions for its replication. Viroid RNA is replicated by the DNA-dependent RNA polymerase of the plant host; preemption of this enzyme may contribute to viroid pathogenicity.

The RNAs of viroids have been shown to contain inverted repeated base sequences at their 3′ and 5′ ends, a characteristic of transposable elements (see Chapter 7) and retroviruses. Thus, it is likely that they have evolved from transposable elements or retroviruses by the deletion of internal sequences.

The general properties of animal viruses pathogenic for humans are described in Chapter 29. Bacterial viruses are described in Chapter 7.

PRIONS

A number of remarkable discoveries in the past 3 decades have led to the molecular and genetic characterization of the transmissible agent causing scrapie, a degenerative central nervous system disease of sheep. Studies have identified a scrapie-specific protein in preparations from scrapie-infected brains of sheep which is capable of reproducing the symptoms of scrapie in previously uninfected sheep. Attempts to identify additional components, such as nucleic acid, have been unsuccessful. To distinguish this agent from viruses and viroids, the term **prion** was introduced to emphasize its proteinaceous and infectious nature. The cellular form of the prion protein (PrP^{c}) is encoded by the host's chromosomal DNA. PrP^{c} is a sialoglycoprotein with a molecular weight of 33,000–35,000 and a high content of α-helical secondary structure that is sensitive to proteases and soluble in detergent. PrP^{c} is expressed on the surface of neurons via a glycosylphosphatidyl inositol anchor in both infected and uninfected brains. An abnormal isoform of this protein (PrP^{res}) is the only known component of the prion and is associated with transmissibility. This abnormal isoform differs physically from the normal cellular isoform by its high beta-sheet content, its insolubility in detergents, its propensity to aggregate, and its partial resistance to proteolysis. A hypothetical protein dubbed "protein X" is believed to interact with a region of PrP^{c} and aid in its conversion to PrP^{res}.

There are additional prion diseases of importance. Kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker disease, and fatal familial insomnia affect humans. Bovine spongiform encephalopathy (BSE), which is thought to result from the ingestion of feeds and bone meal prepared from rendered sheep offal, has been responsible for the deaths of more than 170,000 cattle in Great Britain since its discovery in 1985. A new variant of CJD has been associated with human exposure to BSE in the UK and France. A common feature of all of these diseases is the conversion of a host-encoded sialoglycoprotein to a protease-resistant form as a consequence of infection.

Exhibit B

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Epidemiologic Background of Hand Hygiene and Evaluation of the Most Important Agents for Scrubs and Rubs

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INTRODUCTION

Nosocomial infections (NIs) remain a major global concern. Approximately 2 million NIs occur annually in the United States (232). Overall national prevalence rates have been described as ranging between 3.5 and 9.9% (160), but they vary significantly between departments, patient groups, types of surgical procedures, and the use of indwelling medical devices, etc. (20, 162). The most common NIs are urinary tract infections, lower respiratory tract infections, surgical-site infections, and primary septicemia (27, 159, 528, 532). They lead to additional days of treatment (146, 232, 411, 431, 605), increase the risk of death (27, 157), and increase treatment costs (217, 232, 234, 414, 431, 440, 460, 489, 605). The overall financial burden incurred by NIs has been estimated to be $4.5 billion per year in the United States alone (232). Approximately one-third of all NIs are regarded as preventable (193).

In 2002, a new Centers for Disease Control and Prevention (CDC) guideline for hand hygiene in health care settings, entitled *Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force*, was published (71). It provides health care workers with a review of data on hand washing and hand antisepsis in health care settings and provides specific recommendations to promote improved hand hygiene practices and reduce the transmission of pathogenic microorganisms to patients and personnel in health care settings. As a clinical guideline, its chief aim is to reduce the incidence of NIs by providing detailed recommendations on two main aspects of hand hygiene: (i) choice of the most appropriate agents for hand hygiene in terms of efficacy and dermal tolerance and (ii) different strategies to improve compliance in hand hygiene, including hand hygiene practices among health care workers, behavioral theories, and methods for reducing adverse effects of agents. Our review is intended to support the CDC guideline by presenting specific additional aspects of the various agents, such as a broader evaluation of the in vitro and in vivo efficacy in various test models and their mode of action, resistance potential, and effect on compliance in hand hygiene.

Hand hygiene has been considered to be the most important tool in NI control (403, 462) ever since Semmelweis observed its immense effect on the incidence of childbed fever (473). Health care workers have three opportunities for the postcontamination treatment of hands: (i) the social hand wash, which is the cleaning of hands with plain, nonmedicated bar or liquid soap and water for removal of dirt, soil, and various organic substances; (ii) the hygienic (Europe) or antiseptic (United States) hand wash, which is the cleaning of hands with antimicrobial or medicated soap and water ("scrub"); most antimicrobial soaps contain a single active agent and are usually available as liquid preparations; and (iii) the hygienic hand disinfection (Europe), which normally consists of the application of an alcohol-based hand rub into dry hands without water.

For the preoperative treatment of hands two options are available: (i) the surgical hand wash (Europe) or surgical hand scrub (United States) which is the cleaning of hands with antimicrobial soap and water; and (ii) the surgical hand disinfection (Europe), which is the application of an alcohol-based hand rub into dry hands without water.

Three main types of preparations can be used for the different procedures of hand hygiene. (i) The first is plain, nonmedicated soap (social hand wash). (ii) The second is medicated soap (antiseptic and surgical hand wash). The most commonly used agent is chlorhexidine, usually at a concentration of 4 or 2%. Triclosan can also be found in medicated soaps, usually at a concentration of 1%. Hexachlorophene has now been banned worldwide because of its high rate of dermal absorption and subsequent toxic effects, especially among newborns (84, 98). Levels of 0.1 to 0.6 ppm in blood were found among health care workers who regularly used a 3% hexachlorophene preparation for hand washing (323). These findings speak strongly against the topical use of this active agent. The Food and Drug Administration classifies this agent as not being generally recognized as safe and effective for use as an antiseptic hand wash (21). Hexachlorophene is therefore not included in this review. Other active agents such as povidone iodine have rarely been used for the postcontamination treatment of hands and therefore are also not addressed in this review. (iii) The final type is the alcohol-based hand rub (hygienic and surgical hand disinfection). This is a leave-on preparation and this applied to the skin without the use of water.

In addition, non-alcohol-based waterless antiseptic agents are available for use by health care workers. Some of these contain quaternary ammonium-type compounds. They were not discussed in the CDC hand hygiene guideline because

TABLE 1. Contamination rates of health care workers' hands with nosocomial pathogens and their persistence on hands and inanimate surfaces[a]

Pathogen	Contamination rate(s) of health care workers' hands (%) (references)	Duration of persistence on hands (references)	Duration of persistence on inanimate surfaces (references)
Acinetobacter spp.	3–15 (132, 335, 519)	≥150 min (33)	3 days–5 mo (166, 233, 387, 393, 596, 598)
B. cereus	37 (569)	Unknown	Unknown
C. difficile	14–59 (362, 491)	Unknown	≥24 h (vegetative cells), up to 5 mo (spores) (363)
E. coli	Unknown	6–90 min (33, 151)	2 h–16 mo (3, 111, 190, 350, 376, 393, 509)
"Gram-negative bacteria"	21–86.1 (4, 7, 166, 187, 271, 302, 378)	Unknown	Unknown
Influenzavirus, parainfluenzavirus	Unknown	10–15 min (25, 46)	12–48 h (46, 72, 433, 614)
HAV	8–23.8 (11)	Several hours (354, 355)	2 h–60 days (1, 2, 356)
HCV	17 (81)	Unknown	Unknown
Klebsiella spp.	Up to 16.9 (378, 412, 542)	Up to 2 h (33, 81, 151, 514)	2 h–30 mo (111, 190, 376, 393, 509)
MRSA	Unknown	Unknown	4 wk–7 mo (114, 581)
P. vulgaris	1.3–25 (53, 119, 144, 420, 607)	≥30 min (33)	1–2 days (376)
Pseudomonas spp.	Up to 65 (191, 457)	30–180 min (33, 119)	6 h–16 mo (111, 178, 190, 393, 509)
Rhinovirus	19.5–78.6 (490)	Unknown	2 h–7 days (456, 497)
Rotavirus	Unknown	Up to 260 min (22)	6–60 days (1, 2, 24)
Salmonella spp.	15.4–24 (90, 492)	≤3 h (427)	6 h–4.2 yr (209, 376, 467)
S. marcescens	10.5–78.3 (90, 101, 179, 359, 378, 412, 546)	≥30 min (33)	3 days–2 mo (111, 376)
S. aureus	Up to 41 (202)	≥150 min (33)	4 wk–7 mo (190, 394, 509, 581, 582)
VRE	23–81 (90, 112, 221, 378, 541)	Up to 60 min (402)	5 days–4 mo (39, 393, 394, 402, 599)
"Yeasts," including *Candida* spp. and *Torulopsis glabrata*		1 h (79, 564)	1–150 days (65, 452, 564)

[a] Persistance of nosocomial pathogens on inanimate surfaces is important because of the high rate of acquisition of these pathogens on the hands after contact with environmental surfaces (58).

there was insufficient evidence at the time to promote their use; therefore, they are not further evaluated here.

This review provides an in-depth comparison of the several options for hand hygiene, with the aim of further supporting the CDC guideline on hand hygiene.

TYPES OF SKIN FLORA

Three principal types of skin flora have been described. The resident and transient flora were already distinguished in 1938 (447, 470). In addition, the infectious flora was described, with species such as *Staphylococcus aureus* or beta-hemolytic streptococci, which are frequently isolated from abscesses, whitlows, paronychia, or infected eczema (475).

The resident flora consists of permanent inhabitants of the skin. They are found mainly on the surface of the skin and under the superficial cells of the stratum corneum (379). These bacteria are not regarded as pathogens on intact skin but may cause infections in sterile body cavities, in the eyes, or on nonintact skin (292). Resident skin bacteria survive longer on intact skin than do gram-negative transient species (325). The protective function of the resident flora, so-called colonization resistance, has been demonstrated in various in vitro and in vivo studies. Its purpose is twofold: microbial antagonism and the competition for nutrients in the ecosystem (12). Nevertheless, the interactions between bacteria and fungi on the skin are still inadequately understood. Many such interactions have been demonstrated experimentally. Their contribution—which is thought to be a major mechanism of preventing the adherence of pathogens—to the stability of the dermal ecosystem, however, remains unclear (375).

The dominant species is *Staphylococcus epidermidis*, which is found on almost every hand (311, 454, 522). The incidence of oxacillin resistance among isolates of *S. epidermidis* is up to 64.3% (311) and is higher among health care workers who have direct contact with patients than in those who do not (522). Other regular residents are *Staphylococcus hominis* and other coagulase-negative staphylococci, followed by coryneform bacteria such as propionibacteria, corynebacteria, dermabacteria, and micrococci (137, 315, 401). Among fungi, the most important genus of the resident skin flora is *Pityrosporum* (*Malassezia*) (201). Viruses are usually not resident on the skin but can proliferate within the living epidermis, where they may induce pathological changes (361).

Total counts of bacteria on the hands of medical staff have ranged from 3.9×10^4 to 4.6×10^6 (294, 309, 338, 447). Their number increases with the duration of clinical activities, on average by 16 cells per min (438). Some clinical situations are associated with a higher bacterial load on the hands of health care workers: direct contact with patients, respiratory tract care, contact with body fluids, and after being interrupted while caring for a patient (438). In general, however, it is difficult to clearly assign a specific risk of hand contamination to certain patient care activities. Nurses can contaminate their hands with 100 to 1,000 CFU of *Klebsiella* spp. during "clean activities" (81), while 10 to 600 CFU/ml can be found on nurses' hands after touching the groins of patients heavily contaminated with *Proteus mirabilis* (129). In intensive care units (ICU), the number of direct contacts between the hands of the health care workers and the patients is particularly high, leading to a higher risk of NI (148).

The transient skin flora consists of bacteria, fungi, and viruses that may be found on the skin only at times (447). They usually do not multiply on the skin, but they survive and occasionally multiply and cause disease (15). They may come from patients or inanimate surfaces. Between 4 and 16% of the hand surface is exposed by a single direct contact, and after 12 direct contacts, up to 40% of the hand surface may have been touched (74). The transmissibility of transient bacteria depends on the species, the number of bacteria on the hand, their survival on skin, and the dermal water content (230, 344, 418).

In addition, there is the temporary resident skin flora, which

TABLE 2. Overview of NIs traced to the hands of an individual health care worker or another relevant point source and analysis of the main reason for transmission

Pathogen	Type and no. of NIs	Department	Source	Reason for transmission	Reference
Adenovirus	Epidemic keratoconjunctivitis, 126	Ophthalmology	Infected doctor	Carrier (hand)	235
C. tropicalis	Surgical site infections, 8	Cardiothoracic surgery	Surgical nurse	Carrier (hand), use of nonmedicated soap before surgery due to intolerance of the antiseptic soap	226
HCV	Hepatitis C, 5	Orthopedic and general surgery	Infected anesthesist	Wound on finger during incubation	469
K. aerogenes	Urinary tract infections, 17	Urology	Nurse	Carrier (hand)	82
MRSA	Diarrhea, 8	Orthopedic surgery	Health care worker	Carrier (nose and hands)	507
MRSA	Surgical-site infections, 3	Pediatric cardiovascular surgery	Surgeon	Carrier (nose and hands)	595
MRSA	Surgical-site infections, 5	Cardiac surgery	Hand of assisting surgeon	Dermatitis on hand of surgeon	589
S. liquefaciens	Bloodstream infections, 15	Hemodialysis	Contaminated medicated soap	Transient hand carriage, leading to contamination of epoetin alpha	183
S. marcescens	Septicemia, meningitis, pneumonia, 14	Neonatal ICU	Contaminated triclosan-based liquid soap	Use of soap, resulting in transient hand carriage	574
S. marcescens	Septicemia, meningitis, 15	Neonatal ICU	Contaminated brush	Use of the brush, probably resulting in transient hand carriage	16
S. marcescens	Pneumonia, septicemia, urinary tract infection, surgical-site infection, 83	11 different units	Contaminated liquid soap	Use of soap, resulting in transient hand carriage	492
S. marcescens	Surgical-site infections, 5; septicemia, 2	Cardiovascular surgery	Surgical nurse	Highly contaminated nail cream	417
S. aureus	Dermatitis exfoliativa, 42	Obstetrics	Midwife	Hand eczema	102
S. epidermidis	Surgical-site infections with mediastinitis, 7	Cardiovascular surgery	Hand of assisting surgeon	Chronic dermatitis on hand of surgeon	292

persists and multiplies for a limited period on the skin. The definition is more or less identical to that of transient skin flora, because the duration of residence on human skin is uncertain and variable but never permanent (5). In addition, the temporary resident skin flora often includes nosocomial bacteria and fungi (5, 201, 399, 400).

MICROBIAL AND VIRAL FLORAS OF HANDS AND THEIR EPIDEMIOLOGIC ROLE

Gram-Positive Bacteria

Role in NIs. *S. aureus* is the most common gram-positive bacterium causing NIs (353, 533). Its frequency among all pathogens in NIs varies between 11.1 and 17.2% (265, 484, 493, 583). Methicillin resistance in *S. aureus* (MRSA) is increasing worldwide (113, 503, 578), leading not only to NIs but recently also to community-acquired infection. In 139 ICUs in Germany, 14.3% of all 1,535 NIs due to *S. aureus* have been caused by MRSA. This proportion is highest for urinary tract infections (26.4%), followed by primary septicemia (23.3%), and lower respiratory tract infection (12.9%) (161). The most common type of NI caused by *S. aureus* is the surgical-site infection (245, 259, 422).

Enterococcus spp. are isolated in up to 14.8% of patients with NI (484). The most common species are *Enterococcus faecium* and *E. faecalis* (385), which frequently cause urinary tract infections (533). The emergence of vancomycin resistance among enterococci (VRE) has led to an increased recognition of cross-transmission of VRE, including the role of health care workers' hands (29, 347).

Coagulase-negative staphylococci, such as *S. epidermidis*, mainly cause catheter-associated primary bloodstream infections. In ICUs, approximately one-third of all blood culture isolates from patients with nosocomial bloodstream infections were found to be coagulase-negative staphylococci (463, 533).

Frequency of colonized hands. Colonization of health care workers' hands with *S. aureus* has been described to range between 10.5 and 78.3% (Table 1). Up to 24,000,000 cells can be found per hand (33). The colonization rate with *S. aureus* was higher among doctors (36%) than among nurses (18%), as was the bacterial density of *S. aureus* on the hands (21 and 5%, respectively, with more than 1,000 CFU per hand) (101). The carrier rate may be up to 28% if the health care worker contacts patients with an atopic dermatitis which is colonized by *S. aureus* (608, 609). MRSA has been isolated from the hands of up to 16.9% of health care workers. VRE can be found on the hands of up to 41% of health care workers (Table 1).

Role of hand colonization in cross-transmission. Hand carriage of pathogens such as *S. aureus*, MRSA, or *S. epidermidis* has repeatedly been associated with different types of NI (Table 2) (212, 455). The analysis of outbreaks revealed that dermatitis on the hands of health care workers was a risk factor for colonization or for inadequate hand hygiene, resulting in various types of NI (Table 2).

Transmissibility of VRE has also been demonstrated. The hands and gloves of 44 health care workers were sampled after

care of VRE-positive patients. Gloves were VRE positive for 17 of 44 healthcare workers, and hands were positive for 5 of 44, even though they had worn gloves (553). One health care worker was even VRE positive on the hands although the culture from the glove was negative (553).

Survival on hands and surfaces. *S. aureus* can survive on hands for at least 150 min; VRE survives on hands or gloves for up to 60 min (Table 1). On inanimate surfaces, *S. aureus* and MRSA may survive for 7 months, with wild strains surviving longer than laboratory strains (Table 1). VRE may survive on surfaces for 4 months. The long survival on surfaces, together with the relatively short survival on hands, suggests that contaminated surfaces may well be the source of transient colonization despite negative hand cultures.

Gram-Negative Bacteria

Role in NIs. *Escherichia coli* is the most common gram-negative bacterium, causing mainly urinary tract infections (265, 463). *Pseudomonas aeruginosa* is also very common, chiefly causing lower respiratory tract infections (265, 463). In the majority of cases, both types of infection are device associated (364, 463, 531) and are often found among patients in ICUs (260). Manual handling of devices such as urinary catheters, ventilation equipment, and suction tubes emphasizes the importance of the hands of health care workers in possible cross-transmission of gram-negative bacteria. Overall, gram-negative bacteria are found in up to 64% of all NIs (463).

Frequency of colonized hands. Colonization rates of gram-negative bacteria on the hands of health care workers have been described as ranging from 21 to 86.1% (Table 1), with the highest rate being found in ICUs (271). The number of gram-negative bacteria per hand may be as large as 13,000,000 cells (33). The colonization may be long-lasting (302). Even in nursing homes, a rate of 76% has been described for nurses hands (610). Colonization with gram-negative bacteria is influenced by various factors. For example, it is higher before patient contact than after the work shift (187). Hands with artificial fingernails harbor gram-negative bacteria more often than those without (207). Higher colonization rates with gram-negative bacteria also occur during periods of higher ambient temperature and high air humidity (358).

Different species of gram-negative bacteria exhibit different colonization rates. For instance, the colonization rate is 3 to 15% for *Acinetobacter baumannii*, 1.3 to 25% for *Pseudomonas* spp., and 15.4 to 24% for *Serratia marcescens* (Table 1). *Klebsiella* spp. were found on the hands of 17% of the ICU staff sampled, with up to 10,000 bacteria per hand (81). Artificial fingernails have been associated with a higher risk for colonization with *P. aeruginosa* (144).

Role of hand colonization in cross-transmission. Transient hand carriage of various gram-negative bacterial species has quite often been suspected to be responsible for cross-transmission during outbreaks resulting in various types of NI (155, 426, 514, 571). Most reports of cross-transmission of specific gram-negative bacteria come from critical-care areas, such as neonatal ICUs and burn units. Contaminated hands (Table 1), brushes, contaminated plain soap, and contaminated antiseptic soap have been associated with various types of NI, which were quite often caused by *S. marcescens* (Table 2).

Survival on hands and surfaces. Most gram-negative bacteria survive on the hands for 1 h or more. Survival on inanimate surfaces has been reported to be different for the different gram-negative species, with most of them surviving for many months (Table 1). In general, gram-negative bacteria survive for longer on inanimate surfaces than on human skin (151).

Spore-Forming Bacteria

Role in NIs. The main spore-forming bacterium causing NIs is *Clostridium difficile*. It is estimated that between 15 and 55% of all cases of nosocomial antibiotic-associated diarrhea are caused by *C. difficile* (40, 374, 567, 613). Patients with diarrhea caused by *C. difficile* have on average 3.6 additional hospital days attributable to the NI, which in the United States costs approximately $3,669 per case or $1.1 billion per year (289). The overall mortality is 15% (381). Extraintestinal manifestations are very uncommon (≤1%) (156). Patients can be contaminated from, for instance, the hands of hospital personnel and from inanimate surfaces (40).

Frequency of colonized hands. In one study, the hands of 59% of 35 health care workers were *C. difficile* positive after direct contact with culture-positive patients. Colonization was found mainly in the subungual area (43%), on the fingertips (37%), on the palm (37%), and under rings (20%) (362). In another study, 14% of 73 health care worker were culture positive for *C. difficile* on their hands. The presence of *C. difficile* on the hands correlated with the density of environmental contamination (491). During a third outbreak, caused by *Bacillus cereus* in a neonatal ICU, 11 (37%) of 30 fingerprints from health care workers were positive for *Bacillus* spp. (569).

Role of hand colonization in cross-transmission. Transmission of *C. difficile* in an endemic setting on a general medical ward has been shown to occur in 21% of patients, with 37% of them suffering from diarrhea (362). An outbreak of necrotizing enterocolitis among neonates was associated with clostridial hand carriage in four of seven health care workers (173). Another spore-forming bacterium has been described as well: *B. cereus* was transmitted to the umbilicus in 49% of newborns on a maternity ward; the hands of 15% of the health care workers were found to be culture positive (62).

Survival on hands and surfaces. Vegetative cells of *C. difficile* can survive for at least 24 h on inanimate surfaces, and spores survive for up to 5 months (Table 1).

Fungi

Role in NIs. Fungi are less commonly found than bacteria as the causative agent of NIs, but their frequency and importance are increasing (216, 502, 527). In Germany and New Zealand, 6% of all NIs were caused by fungi (397, 484). In Spain, the overall rate was found to be 2.4% in 1990 and 3.2% in 1999, indicating a higher clinical relevance for NIs in the more recent study (26). In the United States, an increase in isolation of yeasts from 7.6 to 10.6% has been noted over a period of 10 years in patients with NIs (593). The most important fungus with respect to NIs is *Candida albicans*. Fungi may cause septicemia, urinary tract infections, or surgical-site infections (463, 500). Device-associated bloodstream infections caused by *Can-*

TABLE 3. Transmissibility of nosocomial pathogens from contaminated hands

Type of pathogen	Contact time (s)	Target	Transmission rate (%)	Reference
C. albicans	Unknown	Hands	69	452
Feline calicivirus	10	Food	18–46	60
		Steel surface	13	
HAV	10	Lettuce	9.2	59
HSV-1	Unknown	Hands	100 (moist skin), 60 (dry skin)	41
Rhinovirus	10	Hands	71	191
Rotavirus	10	Hands	6.6	22
Salmonella spp.	5	Meat	16 (inoculum of 7 cells per fingertip), 100 (inoculum of ≥600 cells per fingertip)	427

dida spp. have become more common among critically ill patients in the last decades (89, 128, 163, 342); the contribution of non-*albicans Candida* spp. is increasingly significant (216). It has also been reported that 21% of all urinary tract infections among ICU patients are caused by *C. albicans* (463).

Frequency of colonized hands. In an ICU, 67 (46%) of the hands of 146 health care workers were colonized with a yeast. The most common species were *Candida* and *Rhodotorula* spp. Respiratory therapists were found to have the highest colonization rate (69%) (221). In another study of nurses and other hospital staff, 75% of the nurses and 81% of the other hospital staff were colonized with a yeast (541). In a long-term-care facility, 41% of 42 health care workers were found to have *Candida* spp. on their hands (378). Yeasts quite often also colonize artificial fingernails (207). Acquisition of *C. albicans* on the hands of health care workers immediately after attending systemically infected patients was reported to occur in 2 of 17 nurses (79).

Role of hand colonization in cross-transmission. Only a few studies are found in the literature which demonstrate the role of hands in cross-transmission (Table 2), sometimes despite negative hand cultures (572). The analysis of an outbreak revealed that caring for a patient who is colonized with *Candida parapsilosis* can lead to positive hand cultures and finally to severe infections or colonization among patients (501). The transmissibility of yeasts from hand to hand is high (Table 3).

Survival on hands and surfaces. On fingertips, only 20% of viable cells of *C. albicans* and *C. parapsilosis* remain detectable after 1 h (79, 564). *Candida* spp. can survive on surfaces for up to 150 days (452, 564). During this period of survival, most yeast cells die within the first few minutes (452).

Viruses

Role in NIs. Viruses account for approximately 5% of all NIs. On pediatric wards, the proportion is higher at 23% (6). Five main groups of viruses have been identified with respect to their nosocomial transmission: blood-borne viruses (e.g., hepatitis B virus [HBV], hepatitis C virus [HCV], and human immunodeficiency virus [HIV]), respiratory route viruses (e.g., respiratory syncytial virus [RSV], influenza virus, rhinovirus, coronavirus, and adenovirus), fecal-oral route viruses (e.g., rotavirus, small round structured viruses [noroviruses], enteroviruses, and hepatitis A virus [HAV]), herpesviruses obtained from direct contact with skin, mucous membranes, or wounds (e.g., herpes simplex viruses, varicella zoster virus, cytomegalovirus, and Epstein-Barr virus), and exotic viruses such as viral hemorrhagic fever viruses (Ebola virus, Marburg virus, Lassa fever virus, and Congo Crimean hemorrhagic fever virus) and rabies virus (8). The fingers, especially the pads and tips, are the most likely areas to come into contact with viruses while touching infected people and their bodily substances as well as other contaminated materials (499, 576).

Frequency of contaminated hands. The risk of direct contact with blood and thereby with blood-borne viruses is variable. In general, it must be assumed that a health care worker wears protective gloves if contact with blood is expected. However, there are still clinical situations in which contamination with blood is unexpected. Health care workers in invasive radiology have blood contact in 3% of clinical activities, surgeons have blood contact in 50%, and midwives have blood contact in 71% (48). Surgical gloves should protect from direct contact with blood, but perforations are found on average in 17% of gloves, which correlates with the detection of blood under surgical gloves in 13% of surgeons (392). Perforations in most gloves (83%) remain undetected by the surgeon (557). Up to 82.5% of protective gloves have invisible perforations (276). In an acute viremic state, HBV may be present in blood at a concentration of 5×10^8 IU per ml of blood (623). A 1-μl volume of blood, which is hardly visible on a hand, may still contain 500 IU of HBV. For HCV, a concentration of 10^4 to 10^7 IU was found in blood (105). Virus detection on the hands has been investigated in a few studies. In a dialysis unit, 23.8% of samples obtained from health care workers' hands were positive for HCV RNA after treatment of HCV-positive patients despite the use of standard precautions, whereas the rate was 8% after treatment of HCV-negative patients (11).

Viruses from the respiratory tract are often found on hands, e.g., rhinoviruses in up to 65% from persons with a common cold (191, 457). Adenovirus has been found on the hands of healthcare workers during outbreaks of keratoconjunctivitis (380) and was isolated from the hands of 46% of patients with epidemic keratoconjunctivitis (35), which emphasizes the potential of virus transfer to hospital personnel through casual hand contact. No data were available regarding the detection of severe acute respiratory syndrome (SARS) virus on hands during the outbreaks in Asia and Canada in 2003.

Rotaviruses can be found on the hands in up to 78.6% of individuals sampled (Table 1) and also on surfaces with frequent hand contact, e.g., TV sets, toys, and patient charts (9). At the peak of a bout of rotavirus gastroenteritis, every gram of

TABLE 4. Spectrum of antimicrobial activity of procedures for hand hygiene derived from the etiology of NIs, data on the transient flora of health care workers' hands, and their role in the transmission of nosocomial pathogens

Type of antimicrobial activity	Required activity	Optional activity[a]
Bactericidal	+	
Mycobactericidal		+
Sporicidal		+
Fungicidal (yeasts)	+	
Fungicidal		+
Virucidal (enveloped viruses)	+	
Virucidal (including nonenveloped viruses)		+

[a] May be relevant in special patient care or during outbreaks.

feces may contain more than 10^7 to 10^8 infectious viral particles (590).

Cytomegalovirus has been isolated from the hands of day care workers (224), but exotic viruses such as hemorrhagic fever viruses have to date not been detected on health care workers' hands.

Role of hand colonization in cross-transmission. Hands play a major role especially in the transmission of blood-borne, fecal, and respiratory tract viruses. The transmission of some viruses from the hands of health care workers has been described (Table 2). In addition, transient hand carriage is associated with the transmission of many viruses, such as rhinovirus (99, 191), RSV (194, 488), astrovirus (136), and cytomegalovirus (109). For the SARS virus, a similar correlation has been described, since hand hygiene was found to be the second most effective measure to prevent cross-transmission of the SARS virus in a hospital (510). Most viruses are easily transmitted from hand to hand, food, or surfaces (Table 3).

Persistence of infectivity on hands and surfaces. Persistance of viruses on the hands has been investigated mainly for fecal and respiratory tract viruses. Artificial contamination of hands with HAV led to an immediate-recovery rate of 70.5% (59). HAV persisted for several hours on human hands (354, 355). With poliovirus, the immediate-recovery rate was 22% but the whole inoculum was recovered after 150 min, indicating an almost complete persistence of poliovirus on hands (505). Rotavirus has been described as persisting on hands for up to 260 min, with 57% recovery after 20 min, 42.6% recovery after 60 min, and 7.1% recovery after 260 min (22). It can be transferred from contaminated hands to clean hands, with 6.6% of the viral contamination transferred 20 min after contamination (Table 3), and 2.8% of the viral contamination transferred 60 min after contamination (22). Rotavirus has been described to persist better on hands than rhinovirus or parainfluenzavirus (24).

Many enveloped viruses such as influenza virus, parainfluenza virus (Table 1), and cytomegalovirus (139) may survive on the hands for 10 to 15 min or even up to 2 h (herpes simplex virus type 1 [Table 1]). Adenoviruses have been described to persist on human skin for many hours (499).

Only a few studies of the persistence of viruses on surfaces have been performed. Rotavirus and HAV can persist for up to 60 days (Table 1) depending on the room temperature, air humidity, and type of surface (495). HIV remains infective on surfaces for up to 7 days, depending on the inoculum and the type of preparation (cell-associated virus or cell-free virus). HIV obtained from clinical specimens remains infective for a few days (568). Influenza A virus may persist on steel for up to 48 h; on other materials, such as paper or handkerchiefs, the virus persists for up to 12 h (46). Rhinovirus may persist for up to 7 days (Table 1).

MINIMUM SPECTRUM OF ANTIMICROBIAL ACTIVITY

The new CDC guideline on hand hygiene does not suggest a specific minimum spectrum of antimicrobial activity of a suitable hand hygiene agent (71). However, it can be derived from the etiology of NIs as well as the data on the skin flora of the hands of health care workers and their role in the transmission of nosocomial pathogens (Table 4). A procedure for the postcontamination treatment of hands must have at least bactericidal, fungicidal (yeasts), and virucidal (coated viruses) activity.

The spectrum of activity can be substantiated in suspension tests (474). In principle, suspension tests are suitable to substantiate the spectrum of antimicrobial activity (474). The suggested activity against coated viruses is based on the frequent contamination of health care workers' hands with blood during routine patient care and thereby possibly with blood-borne viruses, such as HCV or HIV, where neither patients nor health care workers can be protected by vaccination. The contamination of hands with blood may not be visible but may still be infective with HCV or HIV for the health care worker or the next patient (123). That is why activity against coated viruses should be included in the minimum spectrum of activity for an active agent for hand hygiene. Uncoated viruses, however, are usually spread from patients with infective gastroenteritis (e.g., caused by noroviruses or rotaviruses), upper and

TABLE 5. Effect of alkali- and detergent-based nonmedicated soaps on human skin[a]

Type of effect	Effect observed with:	
	Alkaline-based soap	Detergent-based soap
Formation of lime soaps	Yes	No
Swelling	Substantial	Small
Dehydration	Moderate	Moderate to strong
Degreasing	Pronounced	Pronounced, depending on the amount of detergent
pH shift to alkaline	Substantial	Preventable
Neutralization capacity	Noticeably retarded	Somewhat retarded
Regeneration of skin pH	Strongly impaired	Slightly impaired
Surfaction	No	Possible

[a] Reprinted from reference 283 with permission of the publisher.

TABLE 6. Effect of a simple hand wash with water alone on various types of artificial transient hand flora

Microorganism	Duration of hand wash	Mean removal rate (log_{10})	Reference
E. coli	10 s	1.0	23
	1 min	2.99	377
	2 min	3.08	377
	4 min	3.39–3.67	377
Klebsiella spp.	20 s	1.7	81
VRE	5 s	0	402
	30 s	0	402
Rotavirus	10 s	0.79	23
	30 s	1.26–1.57	47

lower respiratory tract infections, or keratoconjunctivitis (e.g., caused by adenoviruses). These infections often have typical and visible symptoms. The activity against uncoated viruses can be restricted to a specific clinical area, e.g., in ophthalmology (adenovirus), pediatrics (rotavirus), or oncology (parvovirus) or to outbreaks of specific infectious diseases caused by uncoated viruses. Additional activity against the whole spectrum of fungi (including molds), mycobacteria, and bacterial spores may be relevant in special patient care situations (e.g., in bone-marrow transplant units) or during outbreaks. A procedure for the preoperative treatment of hands should be at least bactericidally and fungicidally (yeasts) effective, since the hands of most health care workers' hands carry yeasts and since surgical-site infections have also been associated with hand carriage of yeasts during an outbreak.

AGENTS FOR REDUCTION OF THE NUMBERS OF PATHOGENS ON HANDS

Nonmedicated Soap (Social Hand Wash)

Normally, nonmedicated soaps are detergent-based products. Those based on esterified fatty acids and sodium or potassium hydroxide are less skin compatible (Table 5). They are available in various forms including bar soaps, tissue, leaflet, and liquid preparations. This cleaning activity can be attributed to the detergent properties.

TABLE 7. Effect of a simple hand wash with plain soap and water on various types of artificial transient hand flora

Microorganism	Duration of hand wash	Mean removal rate (log_{10})	Reference(s)
E. coli	10 s	0.5	23
	15 s	0.6–1.1	406
	30 s	1.37–3	34, 326, 330
	1 min	2.6–3.23	257, 377, 478, 480
	2 min	3.27	377
P. aeruginosa	30 s	2–3	330
Klebsiella spp.	20 s	1.7	81
S. aureus	30 s	0.52–3	34, 318, 330
S. saprophyticus	30 s	2.49	34
Rotavirus	10 s	0.14	23
	30 s	1.17–1.19	47
C. difficile	10 s	2.0–2.4	57
B. atrophaeus	10 s	2.4	594
	30 s	2.3	594
	60 s	2.1	594

TABLE 8. Effect of various agents for hand hygiene on the resident hand flora

Type of agent	Concn (%)	Duration of treatment (min)	Mean removal rate (log_{10})	Reference(s)
Plain soap	NA	2	−0.05	36
	NA	3	0.3–0.57	96
	NA	5	0.3–0.4	208, 305, 326, 471
Chlorhexidine	4	2	0.35–1.0	36, 317, 329
	4	3	0.68–1.75	96, 218, 341, 471, 472
	4	5	0.9–1.6	49, 305, 404, 471
	4	6	1.2	153
	4	10	2.29	404
Triclosan	1	5	0.6	305
	2	2	0.3	36
	2	5	0.8	49
Ethanol	70	2	1.0	328
	70	3	1.32	326
	80	2	1.5	475
	85	3	2.1–2.5	251
	95	2	2.1	317, 320
Isopropanol	60	5	1.7	472
	70	0.5	1.5	36
	70	1	0.7–0.8	475
	70	2	1.2–1.65	36, 328
	70	3	1.5–2.0	475
	70	5	2.1–2.4	475
	80	3	2.3	483
	90	3	2.4	483
n-propanol	60	1	1.1	475
	60	3	0.8–2.9	213, 240, 251, 341
	60	5	2.05–2.9	208, 213, 471, 479

[a] NA, not applicable.

Effect on microorganisms and viruses. (i) Spectrum of activity. Nonmedicated soaps do not contain any active ingredient with an antimicrobial activity apart from preservatives. That is why in vitro data on the antimicrobial activity of nonmedicated soap rarely exist. The first experiments with soft alkaline soap were carried out by Robert Koch. He found out that multiplication of the vegetative cells of *Bacillus anthracis* was completely (dilution of 1:1,000) or partly (dilution of 1:5,000) inhibited (273). A more recent study described a fungistatic effect of a tenside-based soap at dilutions between 1:64 and 1:1,000 against *Trichosporon cutaneum*, *C. albicans*, *Trichophyton rubrum*, *Trichophyton schönleinii*, *Microsporum audouinii*, and *Microsporum canis* (277). With one plain soap, even limited fungicidal activity was described and largely explained by the presence of preservatives (603).

(ii) Testing under practical conditions. The use of plain soap and water reduces the numbers of microorganisms and viruses by mechanical removal of loosely adherent microorganisms from the hands. Many studies are available which address the reduction of the transient hand flora. The most common type of artificial contamination of hands for test purposes in the United States is *S. marcescens* (21), whereas *E. coli* is the main contaminant used in Europe (115). Regarding the transient flora, a reduction between 0.5 and 2.8 log_{10} units can be found within 1 min for *E. coli* (Table 6). Other types of artificial contamination have been used as well, such as VRE, rotavirus, *Klebsiella* spp., or spores of *Bacillus atrophaeus*. A simple hand wash still leads to a mean reduction of up to 2.4 log_{10} units within 1 min (Table 7). There is basically no effect on resident hand flora after a 2-min hand wash; after a 5-min hand wash, a reduction of 0.4 log_{10} unit was found, and after

3 h of wearing gloves, no reduction at all was observed (Table 8).

(iii) In-use tests. The effect of a social hand wash "in real life" has also been studied. Among 224 healthy homemakers, a single hand wash had little impact on microbial counts (mean log counts before hand wash, 5.72 ± 0.99; mean log counts after hand wash, 5.69 ± 1.04) (307). In a study with 11 volunteers who washed their hands for 15 s with water alone 24 times per day for a total of 5 days, a slight increase of the bacterial counts was observed (mean log bacterial counts: prewash, 4.91 ± 0.46; postwash, 5.12 ± 0.44); when bar soap was used, a similar result was found (mean log bacterial counts: prewash, 4.81 ± 0.46; postwash, 5.07 ± 0.47) (299). Other authors, too, have found paradoxical increases in bacterial counts on the skin after hand washing with plain soap (299, 371, 611). In contrast, another study showed that a 5-min hand wash with regular bar soap reduced the resident hand flora by 0.33 $\log_{10}$ units (326). The use of a nonmedicated soap by a surgical nurse for the preoperative treatment of hands even led to eight cases of surgical-site infection after cardiac surgery, which underscores the limited efficacy of nonmedicated soap (226).

Some studies have examined only microorganisms that are left on the hands after a hand wash. Washing hands with soap and water has been described to be ineffective in eliminating adenovirus from the culture-positive hands of a physician and patients, indicating that mechanical removal was incomplete (235). Transient gram-negative bacteria remained on the hands of health care workers in 10 of 10 cases despite five successive hand washes with soap and water (187). Furthermore, transmission of gram-negative bacteria from hands has been shown to occur 11 of 12 cases when a simple hand wash is carried out (129).

(iv) Risk of contamination by a simple hand wash. One risk of using soap and water is the contamination of hands by the washing process per se. This has been reported for *P. aeruginosa* (143). A possible source is the sink itself, when splashes of contaminated water come in contact with the hand of the health care worker (119). The reason is that the microorganisms are not killed during the hand wash but only removed and distributed in the immediate surroundings of the person, including the clothes. Nonmedicated soaps may also become contaminated and lead to colonization of the hands of personnel and to NIs, e.g., with *S. marcescens* (492) or *Serratia liquefaciens* (183).

Although the data involving nonmedicated soap suggest that a simple hand wash has some effect on the transient hand flora, it must be borne in mind that, in reality, a simple hand wash often does not last longer than 10 s (121, 145, 176, 177, 180, 300, 334, 450, 552).

Effect on human skin. Each hand wash detrimentally alters the water-lipid layer of the superficial skin, resulting in a loss of various protective agents such as amino acids and antimicrobial protective factors. Regeneration of the protective film may be insufficient if many hand washes are carried out in a row. This may lead to damage of the barrier function of the stratum corneum by inhingement of intercellular putty substances. The transepidermal water loss (TEWL) increases, and the skin becomes more permeable for toxic agents. At the same time, the superficial skin cells dry out, resulting in dehiscence of the stratum corneum, initially on the microscopic level and in due course on the macroscopic level (280).

The incidence with which simple soaps and detergents affect the condition of the skin of health care workers' hands varies considerably (407). For years, natural soaps that have high pH values were thought to be more irritating to the skin than synthetic detergents with neutral or acidic pHs. However, subsequent studies have found that pH is less important than other product characteristics as a cause of skin irritation (200). In some studies, plain soaps have caused less skin irritation than synthetic detergents, while in others, plain bar soap caused greater skin irritation than did a synthetic antimicrobial-containing detergent (299, 565). Synthetic detergents also vary in their propensity to cause skin irritation (200, 407). The incidence of detergent-related-irritant contact dermatitis is affected by various factors: the concentration of the compound, the type of detergent (anionic, cationic, amphoteric, or nonionic) and its quantity, the refattening, the vehicle, the time of exposure, and area exposed (50, 133, 283, 565). For example, it has been shown in vivo that higher concentrations of sodium lauryl sulfate (a detergent) caused greater skin irritation than lower concentrations did (133). In addition, anionic detergents are known to cause greater skin irritation than amphoteric or nonionic detergents (565).

Another factor is the temperature of the water that is used for the hand wash. Hot water leads to greater skin irritation, as reflected by in vivo measurements of TEWL and in vitro measurements of the penetration of detergent through the skin (50, 133, 405). This is explained by an increased penetration of detergents into the epidermis (405). In addition, scaling of the skin is greater when hands are washed with hot water (50). Only skin hydration does not appear to be affected by higher water temperatures (50, 405).

Frequent hand washing induces irritative contact dermatitis (ICD) and dry skin (70, 275, 525, 611), which may become colonized with nosocomial pathogens. ICD can be found in 18.3% of nursing staff in hospitals and is a major occupational health concern (523). A single hand wash already significantly reduces the dermal sebum content; the reduction lasts for 1 h. Skin hydration drops at the same time (280). If hands are washed four times within 1 h, the skin does not recover to its normal state within this period (337). In a study with 52 volunteers who washed their hands 24 times per day for a total of 5 days, a significant increase of the TEWL was observed, indicating that the skin barrier function is impaired (299). The prevalence of ICD caused by hand washing with antimicrobial soaps (detergents) is related to the factors listed above (540). The hardness of water may also affect the incidence of ICD due to frequent hand washing (591).

In summary, plain soap has basically no antimicrobial activity. A simple hand wash can reduce transient bacteria by 0.5 to 3 $\log_{10}$ units but has no real effect on the resident hand flora. The dermal tolerance is rather poor (Table 9).

Chlorhexidine

Chlorhexidine is a cationic biguanide (485) and was first established as an antimicrobial agent in 1954 (104). It exists as acetate (diacetate), gluconate, and hydrochloride salts (485). Chlorhexidine gluconate is commonly used either at 0.5 to

TABLE 9. Comprehensive evaluation of the most important agents for hand hygiene[a]

Criterion for evaluation	Effect for:					
	Plain soap (hand wash)	Chlorhexidine (2–4%) (hand wash)	Triclosan (1–2%) (hand wash)	Ethanol (60–85%) (hand rub)	Isopropanol (60–80%) (hand rub)	*n*-Propanol (60–80%) (hand rub)
Spectrum of activity						
Bacteria	−	++	++	+++	+++	+++
Mycobacteria	−	(+)	Unknown	+++	+++	+++
Bacterial spores	−	−	−	−	−	−
Yeasts	−	++	++	+++	+++	+++
Dermatophytes	−	−	+	++	Unknown	Unknown
Coated viruses	−	++	Unknown	+++	+++	+++
Uncoated viruses[b]	−	+	Unknown	+[c]	(+)[d]	(+)[d]
Effect on hand flora (mean $\log_{10}$ reduction)						
Transient bacteria (≤1 min)	0.5–3	2.1–3	2.8	2.6–4.5	4.0–6.81	4.3–5.8
Resident bacteria (≤3 min)	≤0.4	0.35–1.75	0.29–0.8	2.4	1.5–2.4	2.0–2.9
Potential for acquired bacterial resistance	−	Moderate	Low	None	None	None
Effect on skin						
Skin hydration	Decrease	Decrease	Decrease	No change	No change	No change
Skin barrier	Impaired	Impaired	Impaired	No change	No change	No change
Skin irritation	Likely	Likely	Possible	Very uncommon	Very uncommon	Very uncommon
Allergy	Uncommon	Possible	Uncommon	Extremely uncommon[e]	None	None
Effect on compliance with hand hygiene	(↓)	(↓)	(↓)	(↑)	↑	↑

[a] +++, effective within 30 s; ++, effective within 2 min; +, effective in >2 min; (+), partially effective; −, not effective.
[b] Poliovirus and adenovirus, test viruses of prEN 14476.
[c] Ethanol at 95% has virucidal activity within 2 min.
[d] Results largely dependent on the test virus.
[e] Individual cases, possibly due to impurities.

0.75% in aqueous solution or in some detergent preparations or at 2 to 4% in other detergent preparations (327, 328). Its activity is greatly reduced in the presence of organic matter (485), natural corks (321), and hand creams containing anionic emulsifying agents (586). Inactivation of chlorhexidine may result in contamination of solutions containing 0.1% chlorhexidine, e.g., with *Pseudomonas* spp. (78).

The main target is the bacterial cytoplasmic membrane (360, 464). After chlorhexidine has caused extensive damage to the cytoplasmic inner membrane, precipitation or coagulation of protein and nucleic acids occurs (487). Damage also occurs to the outer membrane in gram-negative bacteria and the cell wall in gram-positive cells (131, 142, 227, 228, 236). Chlorhexidine also damages the cytoplasmic membrane of yeasts (588) and prevents the outgrowth, but not the germination, of bacterial spores (511). If chlorhexidine is hydrolyzed, small amounts of carcinogenic *para*-chloraniline may develop (87); this chemical has been found even in manufactured chlorhexidine solutions (274). At temperatures above 70°C, chlorhexidine is not stable and may degrade to *para*-chloraniline (171). An upper limit for *para*-chloraniline has been set in the British Pharmacopoeia at 0.25 mg per 100 mg of chlorhexidine (17).

Effect on microorganisms and viruses. (i) Spectrum of activity. The antimicrobial activity of chlorhexidine is dependent on its concentration. At lower concentrations, chlorhexidine has a bacteriostatic effect against most gram-positive bacteria (e.g., at 1 μg/ml), many gram-negative bacteria (e.g., at 2 to 2.5 μg/ml) (100, 195), and bacterial spores (513). At chlorhexidine concentrations of 20 μg/ml or more, a bactericidal effect can be expected as well as activity against yeasts (487). The actual effective concentration against *Burkholderia cepacia* and *S. aureus* varies with different supplements from 0.004 to 0.4% (factor 100), and the actual killing time also varies with different supplements (phenylethanol or edetate disodium) from <15 min to >360 min (465). In most studies, concentrations for rapid inactivation are well in excess of MICs, e.g., for *S. aureus* (103), *E. coli*, *Vibrio cholerae* (237), and yeasts (214). When used in a liquid soap, chlorhexidine usually has a concentration of 4% and exhibits a bactericidal activity against various gram-negative (130) and gram-positive (249) bacteria. In some comparative studies using suspension tests chlorhexidine (4%) was found to be less effective against MRSA than against methicillin-susceptible *S. aureus*, which has raised concerns about the suitability of the active agent in the prevention of transmission of MRSA (93, 192, 249). This concern has been confirmed with enterococci. Against *Enterococcus* species and VRE, chlorhexidine (4%) was found to be essentially ineffective in suspension tests if neutralization of residual activity is excluded (247). In a comparison with a nonmedicated hand wash product, a chlorhexidine-based scrub yielded a lower reduction of different antibiotic-resistant test bacteria such as MRSA, VRE, or high-level gentamicin-resistant enterococci (175). Chlorhexidine has no sporicidal activity (513). The data on mycobactericidal activity are not unambiguous but do indicate the relevance of a threshold concentration of chlorhexidine. In one report, 4% chlorhexidine was described as having very good activity against *Mycobacterium smegmatis* (reduction of >6 $\log_{10}$ units within 1 min) (54), whereas another study

with *Mycobacterium tuberculosis* suggested a low activity of 4% chlorhexidine (reduction of <3 $\log_{10}$ units within 1 min) (55). Chlorhexidine at 1.5% did not reveal sufficient activity against *Mycobacterium bovis* (56), and chlorhexidine at 0.5% had no activity against *Mycobacterium avium*, *Mycobacterium kansasii*, or *M. tuberculosis* within 120 min (466).

Against dermatophytes such as *Trichophyton mentagrophytes*, chlorhexidine (1.5%) has been described as having no activity (56).

Antiviral activity has been described as good against most enveloped viruses, such as HIV, cytomegalovirus, influenza virus, RSV, and herpes simplex virus (284, 441), but the virucidal activity of chlorhexidine against naked viruses such as rotavirus, adenovirus, or enteroviruses is low (391, 498).

In comparison to other active agents, chlorhexidine has been described to be less effective in vitro against various nosocomial pathogens than is benzalkonium chloride or povidone iodine (517).

Overall, chlorhexidine seems to have good residual activity (13, 34, 305, 328, 423, 468, 476), but the residual activity must be assessed with caution. It may be false positive due to insufficient neutralization of chlorhexidine in the test design, leading to bacteriostatic concentrations beyond the actual exposure time. Significant difficulties in effective neutralization in in vitro tests have been described, and may yield false-positive activity data for this active agent (246, 516, 517, 600). In addition, the clinical benefit of such a residual effect has never been shown.

(ii) Testing under practical conditions. A 1-min hand wash with soap containing 4% chlorhexidine has been reported to lead to a mean reduction of *E. coli* of 3.08 $\log_{10}$ units on artificially contaminated hands (478). In a study with 52 volunteers who washed their hands 24 times per day for a total of 5 days, a significant decrease in the number of resident skin bacteria was observed with a 4% chlorhexidine liquid soap (mean reduction of 0.76 $\log_{10}$ unit) compared to nonmedicated bar soap (mean increase of 0.21 $\log_{10}$ unit) and a povidone-iodine soap (mean reduction of 0.32 $\log_{10}$ unit) (299). Under practical conditions with hands artificially contaminated by MRSA, chlorhexidine-based liquid soap was equally effective as simple soap (188, 220). A similar result was reported after contamination of hands with *S. aureus* (577). A reduction of 2.1 to 3 $\log_{10}$ units was found on hands contaminated with *Klebsiella* spp. after a 20-s hand wash with a soap based on 4% chlorhexidine (81). If hands were contaminated with rotavirus and treated with chlorhexidine soap for 10 s, the number of test viruses was reduced by 86.9%, which was significantly lower than the reductions achieved with 70% ethanol (99.8%) and 70% isopropanol (99.8%) (23). Treatment with 4% chlorhexidine soap for 30 s on hands contaminated with rotavirus leads to a similar effect of only 0.27 to 0.5 $\log_{10}$ unit (47). Under practical conditions and in terms of removal rate from hands, the efficacy against bacterial spores (e.g., *B. atrophaeus*) of an antiseptic liquid soap based on chlorhexidine was similar to that of nonmedicated soap, indicating that within 10 s or 60 s, chlorhexidine does not exhibit a significant sporicidal activity (57, 594). The effect of 4% chlorhexidine on the resident hand flora was found to be a reduction of between 0.35 and 2.29 $\log_{10}$ units, depending on the application time (Table 8).

(iii) In-use tests. The in-use studies yield a heterogeneous picture of the efficacy of chlorhexidine. One of the first studies with chlorhexidine was performed in 1955. A hand cream containing 1% chlorhexidine was rubbed into dry hands and led to a substantial reduction is the number of resident skin bacteria after 30 min (386). In another clinical study, 74 health care workers evaluated plain soap and a liquid soap based on 4% chlorhexidine over 4 months in a neurosurgical unit and a vascular surgery ward. Overall hand contamination was found to be significantly lower after the use of plain soap (mean number of CFU, 125) than after the use of chlorhexidine (mean number of CFU, 150) (343). A hand wash with 4% chlorhexidine was reported to be more effective on the total bacterial count under clinical conditions than was a 1% triclosan hand wash (140). In a prospective crossover study over 4 months with plain soap and a 4% chlorhexidine soap among health care workers in two surgical units, plain soap was found to be significantly more effective than chlorhexidine in reducing bacterial counts from the hands of health care workers (343). After contamination of hands with *Klebsiella* spp., a 98% reduction was described in 19 of 23 experiments in which a soap based on 4% chlorhexidine was used (81); this is an almost 2 $\log_{10}$ unit reduction. Chlorhexidine failed to eliminate MRSA from the hands (140). In contrast, gram-negative bacteria were more likely to be eliminated after the use of chlorhexidine (140, 357, 573, 580). The mean resident flora of the hands of surgeons was reduced by a 3-min application of 4% chlorhexidine from 3.5 $\log_{10}$ units (preoperatively) to 3.15 $\log_{10}$ units (postoperatively) in operations lasting less than 2 h. It has been shown that for operations lasting more than 3 h, 4% chlorhexidine was unable to keep the resident skin bacteria below the baseline value (4.5 preoperatively and 5.2 postoperatively) (76).

(iv) Resistance. The definition of chlorhexidine resistance is often based on a report from 1982 in which the MICs of chlorhexidine for 317 clinical isolates of *P. aeruginosa* were analyzed, leading to the suggestion that resistance to chlorhexidine should be reported if the MIC is ≥50 mg/liter (390).

Resistance to chlorhexidine among gram-positive bacterial species is rather uncommon. Among *Streptococcus* and *Enterococcus* species, no chlorhexidine resistance has been demonstrated (42, 231). However, gram-negative bacteria, such as *E. coli* (389), *Proteus mirabilis* (100, 536), *Providencia stuartii* (227, 228, 554), *P. aeruginosa* (390, 556), *P. cepacia* (348), and *S. marcescens* (291), have frequently been reported to be resistant to chlorhexidine. The frequency of resistance for the different species is variable. A total of 84.6% of clinical isolates of *P. mirabilis* must be considered resistant to chlorhexidine (536). Among other gram-negative bacteria, the rate is lower (42, 195). *C. albicans* was found to have a resistance rate of 10.5% (42, 231).

Acquired resistance to chlorhexidine has been reported to occur in *S. aureus* (249) and among many gram-negative bacteria (37, 38, 434) which were isolated after recurrent bladder washouts using 600 mg of chlorhexidine per liter (537, 538) or after addition of chlorhexidine to catheter bags for paraplegic patients (584). Some of the isolates were highly resistant, with chlorhexidine MICs of ≥500 mg/liter (538). The chlorhexidine resistance is quite clearly linked to hospital isolates only. A

selection of 196 environmental gram-negative isolates did not reveal a resistance to chlorhexidine (147).

High chlorhexidine MICs correlate with poor reduction in the number of test bacteria in suspension tests, which highlights the potential hazard (555). The MIC may be as high as 1600 μg/ml and correlates well with a slow and insufficient bacterial reduction in suspension tests, as shown with strains of *Providencia* (539). The resistance may be single (83), but cross-resistance to other anti-infective agents can also occur. Among isolates of *P. aeruginosa* from industry and hospitals, an association between resistance to antibiotics and chlorhexidine has been described (290). The potential for cross-resistance between antiseptic agents and antibiotics must be given careful consideration (443). Various nonfermenting gram-negative bacteria which were isolated from blood cultures of oncology patients were inactivated only with >500 mg of chlorhexidine per liter (210).

Different mechanisms of resistance have been found. The acquired resistance is probably linked to the inner (227) or the outer (551) membrane of bacterial cells, the cell surface (131), or the cell wall (549). It may also be explained by the presence of plasmids which code for chlorhexidine resistance (269) and may therefore be transfered to other bacterial species (486, 619). A change in lipid content or a reduced adsorption of the antiseptic can be excluded as the main mechanism of resistance, as shown with isolates from urinary tract infections caused by *P. mirabilis* (554) and *S. marcescens* (410).

Recurrent exposure of bacteria to chlorhexidine may lead to adaptation and may enhance their resistance. This phenomenen was shown with *S. marcescens*. One example involves repeated exposure to various contact lens solutions containing between 0.001 and 0.006% chlorhexidine, which enabled *S. marcescens* to multiply in the disinfectant solution (154). Repeated exposure of *P. aeruginosa* to 5 mg of chlorhexidine per liter was shown to increase the MIC from <10 to 70 mg/liter within 6 days (556). A similar result was reported with *Pseudomonas stutzeri*, which became resistant (MIC, 50 mg/liter) after 12 days of exposure to chlorhexidine (550). Even with *Streptococcus sanguis*, a clear increase of the chlorhexidine MIC during permanent chlorhexidine exposure was observed (601). In general, higher exposures to chlorhexidine in hospitals were reported to be associated with higher rates of resistance (67). Recently, some isolates of *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* isolated from soap dispensers were reported to multiply in a 1:2 dilution of a 2% chlorhexidine liquid soap; ATCC strains of *K. pneumoniae* and *A. baumannii* multiplied only at higher dilutions (73). The latter report highlights the potential danger for the hospital.

Resistance to chlorhexidine may even result in nosocomial infections. Occasional outbreaks of NIs have been traced to contaminated solutions of chlorhexidine (345). There is one report that a 0.5% chlorhexidine solution which was used to disinfect plastic clamps for Hickman lines and was handled by health care workers who transmitted the adapted bacteria to intravenous lines led to 12 cases of bacteremia with three fatalities (357). In another outbreak, contamination of a disinfectant solution with *Burkholderia multivorans* led to nine cases of surgical site infection (45). Especially when chlorhexidine resistance is endemic in gram-negative bacteria, the use of chlorhexidine-based hand antiseptics may lead to an increase of NIs by the chlorhexidine-resistant species (100).

Effect on human skin. Chlorhexidine gluconate is among the most common antiseptics causing ICD (540). However, the frequency of hand dermatitis associated with chlorhexidine-containing detergents is concentration dependent; products containing 4% chlorhexidine cause dermatitis much more frequently than do those containing lower concentrations (540). However, even preparations with the same concentration of chlorhexidine (4%) may cause skin irritation at different frequencies (398, 508). The differences are presumably due to other components of the various formulations. The relatively large number of reports of dermatitis related to chlorhexidine gluconate was partly explained by the fact that it was one of the most widely used antiseptics. In a survey of over 400 nurses working in several hospitals, detergents containing chlorhexidine were reported to cause skin damage less frequently than was nonantimicrobial soap or other detergents containing antimicrobial agents (298). In one 5-day prospective clinical trial, a detergent containing 4% chlorhexidine gluconate caused less irritation than did plain bar soap (300). Nonetheless, dry skin may occur with repeated exposure to preparations containing 4% chlorhexidine gluconate (339, 398).

The potential for contact allergy has been studied as well. Among eczema patients, 5.4% were found to have a positive skin reaction after a single patch test with 1% chlorhexidine, indicating the presence of an allergic contact dermatitis. Repeated exposure resulted in a sensitization rate of ca. 50% (310). In another study, 15 (2.5%) of 551 patients showed a strong and obviously relevant skin reaction in a single patch test with 1% chlorhexidine (415). Although these studies were carried out with patients and not with health care workers, the results nevertheless indicate the potential for sensitization and allergic contact dermatitis during frequent use. Allergic reactions to the use of detergents containing chlorhexidine gluconate on intact skin have been reported and can be severe, including dyspnea and anaphylactic shock (30, 92, 124, 138, 158, 270, 409, 425, 430, 468, 526, 563). Some cases of contact urticaria have also occurred as a result of chlorhexidine use (141, 617).

In summary, chlorhexidine (2 to 4%) has good activity against most vegetative bacteria, yeasts, and enveloped viruses but limited activity against mycobacteria, dermatophytes, and naked viruses. It has a moderate potential for acquired bacterial resistance. A hand wash with a chlorhexidine-based soap can reduce the number of transient bacteria by 2.1 to 3 $\log_{10}$ units; the effect on the resident hand flora is smaller, with a mean reduction between 0.35 and 2.29 $\log_{10}$ units. The dermal tolerance is rather poor, and anaphylactic reactions have been reported (Table 9).

Triclosan

Triclosan is one of many phenol derivatives (diphenoxyethyl ether) which have been used as a group of active agents since 1815, when coal tar was used for disinfection (222). Ever since, many different derivatives, such as thymol, cresol, and hexachlorophene, have been isolated and synthesized. Some of them have been used in antiseptic soaps for health care workers. Triclosan was introduced in 1965 and has been marketed

as cloxifenol, Irgasan CH 3565, and Irgasan DP 300. It has very good stability (585) and resists diluted acid and alkali (453). The commonly used concentration in antiseptic soaps is 1%.

The mode of action of triclosan was identified some years ago. For decades, it has been assumed that triclosan attacks the bacterial cytoplasmic membrane (372, 458). Since 1998, we have known that it blocks lipid synthesis by inhibition of the enzyme enoyl-acyl carrier protein reductase, which plays an essential role in lipid synthesis (367). Mutation and overexpression of the *fabI* gene—which encodes the enoyl-acyl carrier protein reductase—are able to abolish the blockage of lipid synthesis caused by triclosan (205, 312). The *fabI* gene was first found in *E. coli* (366) and was subsequently also found in various other bacterial species such as *P. aeruginosa* (215), *S. aureus* (203, 520), and *M. smegmatis* (365). Some other bacteria, such as *Bacillus subtilis*, contain orthologous enoyl-acyl carrier protein reductases, namely those encoded by *fabI* and *fabK*, which are not inhibited by triclosan (204, 206). A genetic sequence coding for broad-spectrum resistance to triclosan has been identified (239).

The identification of the specific mode of action has raised concerns about the development of resistance to triclosan (313, 366, 506). A recent study has shown that this concern is valid. Strains of *P. aeruginosa* were exposed to triclosan and subsequently developed multiresistance to various antibiotics, including ciprofloxacin (86). Particular care should be taken in the use of triclosan in ICUs, where *P. aeruginosa* is the most common nosocomial pathogen, causing lower respiratory tract infection (260).

Effect on microorganisms and viruses. (i) Spectrum of activity. In vitro, triclosan exhibits a bacteriostatic effect at lower concentrations (575); at higher concentrations, it has bactericidal activity (560). The activity of triclosan is greater against gram-positive organisms than against gram-negative bacteria, particularly *P. aeruginosa* (238). MIC of triclosan generally range between 0.025 and 4 mg/liter among isolates of *S. aureus* and MRSA (94, 459, 543). The fungicidal activity of triclosan is good and includes yeasts and dermatophytes (459).

(ii) Testing under practical conditions. For artificially contaminated hands, a 1-min hand wash with 0.1% triclosan has been shown to reduce the number of test bacteria by 2.8 $\log_{10}$ units (475), which is essentially identical to the results obtained with nonmedicated soap (257). A soap based on 1% triclosan was found to reduce the resident hand flora within 5 min by 0.6 $\log_{10}$ unit (305). A 2% concentration yielded no major difference at 0.8 $\log_{10}$ unit (49). If hands were contaminated with rotavirus and treated with 2% triclosan for 30 s, the number of test viruses was reduced by 2.1 $\log_{10}$ units (47). On the resident hand flora, 1 or 2% triclosan has only a small effect, showing a mean reduction between 0.29 and 0.8 $\log_{10}$ unit within 5 min (Table 8).

(iii) In-use tests. In comparison to plain soap, at 0.2% triclosan does not further reduce bacterial counts on the hands (295). Under clinical conditions, a hand wash with 1% triclosan was reported to be less effective on the total bacterial count than a 4% chlorhexidine hand wash (140). Triclosan was able to eliminate MRSA from the hands (140). In contrast, gram-negative bacteria were less likely to be eliminated after the use of triclosan (140).

(iv) Resistance. One *S. aureus* isolate for which the triclosan MIC is >6,400 mg/liter has been described (494). Some isolates of gram-negative bacteria have been found with triclosan MICs of >100 mg/liter as well (459). This high resistance was not transferable and was probably chromosomal (494). Exposure of *S. aureus* to 0.01% triclosan over 28 days did not result in a change of the triclosan MIC (543). Using *S. epidermidis* in a similar test, however, resulted in an increase of the MIC from 2.5 to 20 mg/liter, indicating a high potential for adaptation of the bacterium (545). Exposure of *P. aeruginosa* to 25 mg of triclosan per liter yielded multiresistant mutants which exhibited resistance to triclosan (MIC, >128 mg/liter) and some antibiotics, e.g., tetracycline (MIC, >256 mg/liter), trimethoprim (MIC, >1,024 mg/liter), and erythromycin (MIC, >1,024 mg/liter) (86).

An antiseptic hand wash preparation based on 1% triclosan was found to be contaminated with *S. marcescens* in an operating theater and a surgical ICU (43). This involved 4 (17%) of 23 bottles and 5 (28%) of 18 wall dispensers, but no association with a higher rate of NIs was found (43).

The widespread use of triclosan in antibacterial household products such as liquid soaps is cause for concern that selection for bacteria with an intrinsic resistance to triclosan may be occurring (314). Triclosan can be found in 76% of antibacterial liquid soaps in the United States (424), which has led to the recommendation that it should not be used in consumer products (547). It is therefore not surprising that highly resistant bacteria were detected in compost, water, and soil (369). Two species, *Pseudomonas putida* and *Alcaligenes xylosoxidans*, were even capable of metabolizing triclosan and thereby of actively "digesting" the active agent (369).

Effect on human skin. Detergents containing less than 2% triclosan are generally well tolerated. In one laboratory-based study of surgical hand disinfectants, a detergent containing 1% triclosan caused fewer subjective skin problems than did formulations containing an iodophor, 70% ethanol plus 0.5% chlorhexidine gluconate, or 4% chlorhexidine gluconate (305). Allergic reactions to triclosan-based handwash products are uncommon (616).

In summary, triclosan (1 to 2%) has good activity against vegetative bacteria and yeasts but limited activity against mycobacteria and dermatophytes. The activity against viruses is unknown. Triclosan has a low potential for acquired bacterial resistance. A hand wash with a triclosan-based soap can reduce the number of transient bacteria by 2.8 $\log_{10}$ units; the effect on the resident hand flora is lower, yielding a mean reduction between 0.29 and 0.8 $\log_{10}$ unit. The dermal tolerance is rather poor (Table 9).

Ethanol, Isopropanol, and *n*-Propanol

The general antimicrobial activity of alcohols has been described to increase with the length of the carbon chain and reaches a maximum at six carbon atoms (548). Solubility in water has led to a preference for ethanol and the two propanols. Alcohols have a nonspecific mode of action, consisting mainly of denaturation and coagulation of proteins (241). Cells are lysed (229, 428), and the cellular metabolism is disrupted (360).

Ethanol is a well-known antimicrobial agent, which was first

recommended for the treatment of hands in 1888 (473). The antimicrobial activity of isopropanol (equivalent to propan-2-ol) and *n*-propanol (equivalent to propan-1-ol) was first investigated in 1904 (612). Many studies followed and supported the use of the two propanols for hand disinfection (52, 85, 322, 395).

Both the alkyl chain length and branching affect the antimicrobial activity (562). The following ranking regarding the bactericidal activity has been generally established: *n*-propanol > isopropanol > ethanol (95, 476, 548). The bactericidal activity is also higher at 30 to 40°C than at 20 to 30°C (561). In terms of virucidal activity, ethanol is superior to the propanols.

Effect on microorganisms and viruses. (i) Spectrum of activity. *(a) Ethanol.* Ethanol has a strong immediate bactericidal activity (297) that is observed at 30% and higher concentrations (383, 444, 448, 449). Against *S. aureus*, *E. faecium*, or *P. aeruginosa*, its bactericidal activity seems to be slightly higher, at 80% than at 95% (110). According to the tentative final monograph for health care antiseptic products, ethanol is considered to be generally effective at between 60 and 95% (21). The spectrum of bactericidal activity of ethanol is broad (198).

Ethanol is also effective against various mycobacteria. Ethanol at 95% killed *M. tuberculosis* in sputum within 15 s, 70% ethanol required a contact time of 30 s, and 50% ethanol required 60 s (524), which was also required against *M. smegmatis* (54). Similar results were obtained with 70% ethanol and *M. tuberculosis* (55). For *Mycobacterium terrae*, the surrogate test strain for *M. tuberculosis*, a $\log_{10}$ reduction of >4 was found with 85% ethanol within 30 s (258). Very good activity was also shown with 70% ethanol against *M. bovis* (56).

In addition, ethanol has broad activity against most fungi—including yeasts and dermatophytes—at different exposure times and under different test conditions (56, 134, 258, 285, 286, 331).

The spectrum of virucidal activity is largely dependent on the concentration of ethanol. Higher concentrations of ethanol (e.g., 95%) generally have better virucidal activity than do lower concentrations, such as 60 to 80%, especially against naked viruses (127, 244, 534). A hand rub based on 95% ethanol has been described to have broad virucidal activity within 2 min, even against the most common nonenveloped viruses such as poliovirus and adenovirus (19). A gel based on 85% ethanol was still effective with a reduction factor (RF) of >4 against poliovirus within 3 min and against adenovirus within 2 min (258). Most naked viruses such as poliovirus (258, 262, 268, 535, 566), astroviruses (288), feline calicivirus (164), rotaviruses (258, 288), and echoviruses (287, 288) are inactivated by ethanol as well. HAV may be the only virus which is not fully inactivated; however, a higher RF of 3.2 was found with 95% ethanol whereas the RF was only 1.8 with 80% ethanol (615). Preparations containing less than 85% ethanol are usually less effective against viruses (570), although they may reveal sufficient activity within 10 min against various nonenveloped viruses such as adenovirus, poliovirus, echovirus, or Coxsackie virus (268). Under variable test conditions and at different exposure times, ethanol has broad general activity against the enveloped viruses, such as vaccinia virus (61, 184, 185, 268), influenza A virus (185, 268), togaviruses (77), Newcastle disease virus (97), HIV (346, 529), HBV (68, 272), and herpes simplex viruses (268).

Ethanol is known to have virtually no sporicidal activity (56, 165). This was first described over a century ago (135, 199, 395, 461). A pseudo-outbreak was reported due to contamination of ethanol with spores of *B. cereus*. The ethanol was used in the hospital pharmacy for preparation of skin antiseptics without spore filtration (219). Another report described contamination of 70% ethanol with spores of *Clostridium perfringens*, which was eliminated by addition of 0.27% hydrogen peroxide over 24 h (602).

(b) Isopropanol. The bactericidal activity of isopropanol begins at a concentration of 30% (445) and increases with increasing concentration but is lower again at 90% (544). It is similar to the bactericidal activity of *n*-propanol (612). In suspension tests, a hand rub based on propanols (total of 75%, wt/wt) had a comprehensive bactericidal activity against 13 gram-positive species, 18 gram-negative species, and 14 emerging pathogens within 30 s. Test bacteria included both ATCC strains and clinical isolates (248). Variations of the test conditions (e.g., with organic load) usually have no effect on the overall result in suspension tests (253). A tuberculocidal activity was found with isopropanol between 50 and 70% (150). The virucidal activity against naked viruses is limited and usually does not include enteroviruses such as astrovirus or echovirus (287, 288). If the exposure time is extended, sufficient activity against some nonenveloped viruses—such as echovirus (90% isopropanol for 10 min), feline calicivirus (50 to 70% isopropanol for ≥3 min), or adenovirus (50% isopropanol for 10 min)—can be achieved (164, 268). Isopropanol alone has no sporicidal activity, as shown with spores of *B. subtilis* and *Clostridium novyi* (445).

(c) n-Propanol. As early as 1904, *n*-propanol was described as an alcohol with a very strong bactericidal effect (548, 612) starting at a concentration of 30% (250). Compared to isopropanol, the activity against feline calicivirus seems to be better (164). In general, however, the antimicrobial activity of *n*-propanol is thought to be similar to that of isopropanol (475).

(ii) Testing under practical conditions. *(a) Ethanol.* On hands artificially contaminated with *E. coli*, ethanol at concentrations between 70 and 80% caused a reduction in the number of test organisms of between 3.8 and 4.5 $\log_{10}$ units within 60 s (475–477), and 1.96 $\log_{10}$ units within 10 s (23). Significant differences may be observed among alcohol-based gels. Up to an ethanol concentration of 70%, gels have been described to be significantly less effective than the reference hand disinfection (282, 432). A preparation with 85% ethanol, however, was found to be as effective as the reference hand disinfection, with 3 ml within 30 s (258).

Other types of artificial contamination of hands have only rarely been tested. Using *S. aureus*, a 30-s application of 70% ethanol achieved a 2.6 or 3.7 $\log_{10}$ unit reduction (34, 318). A similar result was found with 79% ethanol against *Micrococcus luteus* (mean RF, 3.2 after 30 s) (174). If hands were contaminated with rotavirus and treated with 70% ethanol for 10 s, the number of test virus was reduced by 2.05 $\log_{10}$ units (23). A longer application time of 30 s revealed a similar reduction of 2.72 $\log_{10}$ units (47). Low ethanol concentrations, e.g., 70 or 62%, did not even achieve a 1 $\log_{10}$-unit reduction of HAV on contaminated hands (355) but achieved a 2.9 to 4.2 $\log_{10}$-unit reduction within 20 s against adenovirus, rhinovirus, and rotavirus (496). Contamination with poliovirus was reduced by only

TABLE 10. Baseline compliance rates in hand hygiene, according to the agent

Type of ward(s)	Type of agent(s)[a]	Baseline compliance rate (%)	Reference(s)
Pediatric ICU	Soap	30.1	118
All wards	Soap	30.2	592
Long-term care	Soap	31.9	558
All wards	Soap	32	334
Pediatric ICU	Soap	34	196
Medical ICU	Soap	38.1	10
All wards	Soap	45	293
Surgical ICU	Soap	45	429
ICU and oncology ward	Soap	56	515
Medical ICU	Soap	60.7	266
ICU	Soap	61.4	530
ICU	Soap	63.1	261
Neonatal ICU and infant rehydration unit	Plain soap	29	308
ICU	Medicated soap (4% chlorhexidine)	42	117
ICU	Plain soap and alcohol	38[b]	117
ICU	Plain soap and alcohol	40.2	622
Emergency department	Plain soap and isopropanol (60%)	32.3	370
All	Plain soap and isopropanol (70%)	48	439
ICU	Medicated soap (chlorhexidine) and alcohol	28.7	176
ICU	Alcohol	55.2	126

[a] "Soap" was always assumed to be meant when "hand washing" was mentioned in a study; it may include plain and medicated soap.
[b] Low compliance was explained by incorrect and rare use of alcohol.

1.6 $\log_{10}$ units within 10 s by use of 70% ethanol (534). A solution of 80% ethanol reduced the carriage of poliovirus on fingers by only 0.4 $\log_{10}$ unit within 30 s (106). A higher concentration of ethanol (95%) reduced different naked viruses, such as adenovirus (RF, >2.3), poliovirus (RF, between 0.7 and 2.5), and coxsackievirus (RF, 2.9), significantly better on the hands (504). Against feline calicivirus, a sufficient efficacy (RF $\geq$ 3.83) was observed with 70% ethanol within 1 min without an organic load (164). Experiments with 5% fecal test suspension as the organic load, however, demonstrated a lowered efficacy of ethanol. Within 30 s, ethanol at 70% revealed a mean $\log_{10}$ reduction between 1.27 and 1.56 (244) and ethanol at 95% was more effective (mean RF between 1.63 and 2.17) (244).

The lack of sporicidal efficacy has been recently confirmed under practical conditions of hand contamination, using spores of *B. atrophaeus*, a surrogate for *B. anthracis* (594).

The effect on the resident hand flora depends on the ethanol concentration and the application time. A reduction between 1.0 and 1.5 $\log_{10}$ units has been found with ethanol at 70 and 80% within 2 min; higher concentrations (80 and 85%) and longer application times led to mean reductions between 2.1 and 2.5 $\log_{10}$ units (Table 8).

Comparison to antimicrobial soaps or nonmedicated soaps usually reveals the superior efficacy of ethanol on the resident hand flora or on artificial contamination of hands with *E. coli* or *S. marcescens* (32, 34, 66, 80, 267, 318, 377, 406, 419, 476). To date, there is only one study with a 2-min application time, yielding the opposite result (319). Other test models have been investigated as well. Compared to washing hands with plain soap, a 30-s hand disinfection using 70% ethanol was significantly more effective in reducing the transfer of *Staphyloccocus saprophyticus* (344). The higher bactericidal efficacy of ethanol than of antimicrobial soaps is even more pronounced in the presence of blood (296, 297).

Comparison to other alcohols reveals only minor differences. Using *S. marcescens* as a test organism, 70% ethanol with 0.5% chlorhexidine was described to be more effective under practical conditions than was 70% isopropanol, which may be explained by the different type of alcohol, the additional chlorhexidine, or both (14).

(b) Isopropanol. Isopropanol (60%) has been chosen as the reference agent for testing the efficacy of hygienic hand disinfection in European standard EN 1500 (116). With the reference treatment on hands which were artificially contaminated with *E. coli* and treated with two 3-ml doses for a total of 60 s, a mean reduction of 4.6 $\log_{10}$ units was achieved (256, 257). In other studies, similar results of 4.0 to 4.4 $\log_{10}$ units within 60 s were found (472, 475, 480, 482). The reduction with 70% isopropanol after 10 s, however, is 2.15 $\log_{10}$ units (23). In contrast, a gel based on 60% isopropanol was found to be significantly less effective than three liquid rinses against three test bacteria at 15 and 30 s (110). Using bacteria other than *E. coli* to artificially contaminate hands, similar mean reductions were found after 30 s in *S. aureus* (mean RF, 6.36), *E. faecalis* (mean RF, 6.07), and *P. aeruginosa* (mean RF, 6.81) (110). After 15 s, mean RFs were only marginally lower in *S. aureus* (mean RF, 5.90), *E. faecalis* (mean RF, 5.03), and *P. aeruginosa* (mean RF, 6.05) (110). If hands were contaminated with rotavirus and treated with 70% isopropanol for 10 s, the number of test viruses was reduced by 99.8% (RF, 2.7). The number of *E. coli* cells is reduced to a similar extent (99%; RF, 2.0) (23). A similar result was obtained when hands were contaminated with rotavirus and treated with 70% isopropanol for 30 s. The number of test viruses was reduced by 3.1 $\log_{10}$ units (47). Contamination with poliovirus was reduced only by 0.8 $\log_{10}$ units within 10 s after use of 70% isopropanol (534). The efficacy against feline calicivirus is also quite low, with a mean reduction of 0.76 $\log_{10}$ unit (90% isopropanol) or 2.15 $\log_{10}$ units (70% isopropanol) within 30 s (164).

TABLE 11. Compliance rates in hand hygiene, according to the agent and intervention

Type of ward(s)	Agent(s)[a] (baseline)	Main active agent(s)[a] (new)	Intervention(s)	Compliance rate (%)		Main reason(s) for change	Reference
				Baseline	After intervention		
ICU	Soap	Soap	Lectures, feedback, demonstrations	5	63	Lectures, feedback, demonstrations	51
ICU	Soap	Soap	New design of ICU	16	30	More convenient sink location	446
ICU	Soap	Soap	Education	22.0	29.9	Education	518
ICU	Soap	Soap	Lectures and reminder labels on ventilators	46 (before patient contact), 83 (after patient contact)	92 (before patient contact), 92 (after patient contact)	Lectures and reminder labels on ventilators	264
Pediatric ambulatory setting	Soap	Soap	Use of reminders	49	49	Use of reminders	324
Pediatric wards	Soap	Soap	Educational program	52	74	Educational program	31
Emergency department	Soap	Soap	Posting of signs, education	54	64	Posting of signs, education	120
Neonatal ICU	Soap	Soap	Use of gowns	62	60	Use of gowns	421
ICU	Soap	Soap	Performance feedback and new soap	63	92	Performance feedback	352
ICU	Soap	Soap	Education and feedback	81	92	Education and feedback	122
Surgical ICU	Plain and medicated soap (chlorhexidine)	Plain and medicated soap (chlorhexidine)	Automatic hand-washing machines available	22	38	Availability of hand-washing machines	618
New-burn nurseries	Medicated soap	Medicated soap	Feedback	28	63	Feedback	451
Medical ICU	Medicated soap	Medicated soap	Routine wearing of gowns and gloves, educational meetings	40.8	58.2	Routine wearing of gowns and gloves, educational meetings	521
ICU	Medicated soap (*para*-chloro-metaxylenol)	Medicated soap (*para*-chlorometaxylenol)	Teaching and reminders (buttons)	22	29.9	Reminders	518
Medical ICU	Medicated soap (4% chlorhexidine)	Medicated soap (4% chlorhexidine)	Education	26% (before patient contact), 23% (after patient contact)	38% (before patient contact), 60% (after patient contact)	Education	91
Pediatric ICU	Soap (plain and medicated), ethanol (70%), aqueous povidone iodine	Soap (plain and medicated), ethanol (70%), aqueous povidone iodine	Overt observation and feedback	12% (before patient contact), 11% (after patient contact)	68% (before patient contact), 65% (after patient contact)	Overt observation and feedback	559
ICU	Soap	Ethanol (60%)	Quality improvement and introduction of hand gel	42.5	35.1	"Sticky uncomfortable feeling" of product	197
ICU and medical ward	Plain and medicated soap (4% chlorhexidine)	Ethanol (60%)	Education program followed by introduction of hand rub	16.3	20.9 (after education), 33.2 (after introduction of hand rub)	Easy access of hand rub	63
Medical ICU and ward	Soap	Ethanol (62%)	Introduction of hand rub with an educational and motivational campaign; wall dispensers	Overall, 60%; medical ICU, 70.3%; ward, 46.2%	Overall, 52%; Medical ICU, 58%; ward, 48%	Physicians as role models	388
Plastic surgery department	Soap	Ethanol (70%)	Performance feedback	62	61.3	Performance feedback	336
Neonatal ICU	Plain soap	Ethanol (79%)	Introduction of hand rub and quality improvement	44	48	Unknown	75
ICU	Soap	Isopropanol (60%)	Introduction of hand rub and teaching	32	45	Accessability of hand rub	180
ICU	Plain soap	Isopropanol (75%)	Promotion campaign for hand hygiene	38.4	54.5	Campaign on benefits of alcohol-based hand rubs	223
All		Isopropanol (75%)	Promotion campaign for hand hygiene	48	66	Campaign on benefits of alcohol-based hand rubs	439
Medical ICU	Plain soap	Isopropanol (45%) and *n*-propanol (30%)	Introduction of hand rub and education	42.2	60.9	Availability of hand rub; short time for hand rub procedure	351
All	Soap (plain and medicated)	Isopropanol (45%) and *n*-propanol (30%)	Introduction of hand rub and education	62.2	66.5	Better dermal tolerance of hand rub	167
All	Soap (plain and medicated)	Isopropanol (45%) and *n*-propanol (30%)	Introduction of hand rub and education	52	66	Dermal tolerance and accessibility	169

[a] "Soap" was always assumed to be meant when "hand washing" was mentioned in a study; it may include plain and medicated soap.

Isopropanol at 60 and 70% has a rather low efficacy against the resident hand flora within 2 min (RF, between 0.7 and 1.2). With longer application times (3 and 5 min) and higher concentrations of isopropanol (80 and 90%), the mean reduction of the resident hand flora is between 1.5 and 2.4 (Table 8).

Comparison of isopropanol with nonmedicated soaps and antimicrobial soaps reveals the better efficacy of isopropanol, both on the resident hand flora (129, 316) and on hands which were artificially contaminated (33, 44, 472), with only one study showing discrepant results (306).

(c) n-Propanol. On hands which were artificially contaminated with *E. coli*, n-propanol at 100, 60, or 50% reduced the number of test bacteria within 1 min by 5.8, 5.5, or 5.0 $\log_{10}$ units, respectively (482, 604). Lower concentrations, e.g., 40%, still reduce the test bacteria by 4.3 $\log_{10}$ unit within 1 min (475). The efficacy against feline calicivirus seems to be quite good, with a mean RF between 1.9 (80% *n*-propanol) and ≥ 4.13 (50% *n*-propanol) within 30 s (164). Against the resident hand flora, 60% *n*-propanol is quite effective, with a mean reduction of 1.1 after 1 min and of 2.05 to 2.9 after 5 min (Table 8). A combination of isopropanol (45%) with *n*-propanol (30%) is significantly more efficacious than *n*-propanol (60%) on the resident hand flora in two studies; yielding a mean RF of 4.61 versus 2.9 in one study (240) and a mean RF of 1.45 versus 0.83 in the other (341).

(iii) In-use tests. *(a) Ethanol.* During an outbreak of gentamicin-resistant *Klebsiella aerogenes*, a health care worker was found to carry the strain on her hand. *K. aerogenes* was still detectable on two occasions after use of 95% ethanol for hand disinfection. The nurse continued to carry the strain for almost 4 weeks on her hand (82). Especially among health care workers wearing artificial fingernails, ethanol (60%) was found to be more effective in the removal of nosocomial pathogens than was an antimicrobial soap (368).

(b) Isopropanol. Under clinical conditions, a combination of isopropanol, *n*-propanol, and mecetronium etilsulfate was found to be significantly more effective than a chlorhexidine-based liquid soap (168). Isopropanol at 60% was found to have a better bactericidal efficacy on the resident hand flora than do antiseptic soaps based on chlorhexidine or triclosan (382). The higher bactericidal efficacy of isopropanol compared to antimicrobial soaps is even more pronounced in the presence of blood (296, 297).

Isopropanol at 60 to 70% was found to be necessary for removal of aerobic gram-negative bacteria from hands, whereas a simple hand wash with soap was inadequate (125). Transmission of gram-negative bacteria was also significantly better interrupted by propanol than by a social hand wash following brief contact with a heavily contaminated patient source (129).

(c) n-Propanol. Comparisons of *n*-propanol with nonmedicated soaps and antimicrobial soaps consistently reveal the greater efficacy of *n*-propanol on hands which were artificially contaminated (33, 480, 482). Comparison between *n*-propanol and isopropanol reveals a slightly greater efficacy of *n*-propanol (33). The efficacy of 60% *n*-propanol was found to be similar to that of 90% isopropanol on the resident bacteria (483).

(iv) Resistance. No acquired resistance to ethanol, isopropanol, or *n*-propanol has been reported to date.

Effect on human skin. Alcohols are considered to be among the safest antiseptics available and generally have no toxic effect on human skin (332). One of the first studies was carried out in 1923 and found that isopropanol had no noticeable harmful effect on human skin (181). This has been confirmed in a repetitive occlusive patch test with *n*-propanol at various concentrations (333). In addition, different formulations based on various alcohols were tested on intact skin for 6 days and 4 weeks and were well tolerated (279). The skin barrier remains intact, dermal hydration does not change significantly, and the dermal sebum content remains unchanged (279). A similar result was found in a repetitive occlusive patch test with an ethanol-based hand gel (255) and a propanol-based hand rub (254). Even on preirritated skin, the potential for irritation by commonly used alcohols is very low (333). Repeated exposure to alcohol or a moderately formulated product can cause or maintain skin dryness and irritation (108, 197, 475). Ethanol is less cytotoxic (278) and may be less irritating than *n*-propanol or isopropanol (108, 281, 423). Adding 1 to 3% glycerol, humectants, emollients, or other skin-conditioning agents can reduce or eliminate the drying effects of alcohol (34, 182, 306, 328, 396, 408, 481, 587).

Various studies have addressed the question whether alcohol-based hand rubs have a dermal tolerance that is similar to or better than that of nonmedicated or antimicrobial soaps. Several prospective trials have demonstrated that alcohol-based hand rubs containing emollients may cause significantly less skin dryness and irritation than washing hands with liquid detergents (70, 303, 304, 378, 611). For example, a prospective, randomized clinical trial with crossover design was conducted with nurses working on several hospital wards in order to compare hand washing with a nonantimicrobial liquid detergent and hand disinfection using a commercially available alcohol hand gel. The condition of the skin of nurses' hands was determined at the beginning, midpoint, and end of each phase of the trial by using participants' self-assessment, visual assessment by an observer, and objective assessment of skin dryness via measurements of the electrical capacitance of the skin on the dorsal surface of the hands. Self-assessments and visual assessments by the observer both found that skin irritation and dryness occurred significantly less often when nurses routinely used the alcohol-based hand gel between attending to patients, and electrical skin capacitance readings demonstrated that skin dryness occurred significantly less often when the alcohol hand gel was used (70). A questionnaire study conducted at the end of the trial found that more than 85% of nurses felt that the alcohol hand rub caused less skin dryness than did washing with soap and water and that they would be willing to use the product routinely for hand hygiene (69). In another study of 77 operating-room staff who used either an alcohol-based hand rub or an antiseptic liquid soap for surgical hand disinfection, skin dryness and skin irritation decreased significantly in the group using the alcohol rub whereas they both increased in the group using soap (416). In another clinical trial, nurses were randomly assigned to use either a nonantimicrobial liquid detergent or an alcohol-based hand rinse, and skin tolerance was studied by using a combination of self-assessments, evaluations by a dermatologist, and measurements of TEWL. Self-assessments and those of the dermatologist found that the alcohol hand rinse was tolerated significantly better than the liquid

detergent (611). There was no significant difference in TEWL readings with the two regimens. In a prospective, randomized trial conducted with ICU personnel, the effects on skin condition of a detergent containing 2% chlorhexidine were compared to those of an alcohol-based hand rub. Both the skin scaling scores and self-assessments found that the alcohol-based hand rub was tolerated better than the detergent containing 2% chlorhexidine (303). In a similar randomized, prospective trial in a neonatal ICU, the alcohol-based hand rinse regimen was tolerated significantly better than a detergent containing 2% chlorhexidine (301).

In a prospective intervention trial designed to study the impact of introducing an alcohol hand rinse on hand hygiene compliance among health care workers, dermatologist-assessed skin dryness and irritation revealed that the alcohol hand rinse was tolerated better than the traditional antiseptic hand-washing preparation (167). Measurements of skin hydration improved (although not significantly) after the alcohol hand rinse was introduced. Other clinical studies have also shown that alcohol-based hand rubs are tolerated well by health care workers (351). Furthermore, in a laboratory-based study of hand disinfection which compiled observations by an expert, self-assessments, and TEWL measurements, an alcohol-based hand rub caused less skin irritation than did a detergent containing 2% chlorhexidine (186).

Another trial based only on self-assessments to determine the impact on skin condition of an alcohol hand rub versus a detergent containing 4% chlorhexidine gluconate also found that the alcohol-based product was better tolerated (384).

In health care facilities where hand washing with plain soap or antimicrobial soap and water has been the rule, switching (particularly in the winter) to an alcohol-based hand rub may cause some personnel to complain of burning or stinging of the skin when applying alcohol. This is usually due to the presence of underlying, detergent-associated ICD among personnel (252). Skin that has been damaged by preexisting exposure to detergents may be more susceptible to irritation by alcohols than are nondamaged skin areas (333). As the skin condition improves with continued use of alcohol-based hand rubs, the burning and stinging associated with applying alcohol invariably disappears.

Allergic contact dermatitis or contact urticaria syndrome induced by exposure to alcohol-based hand rubs occurs rarely (88), and the cause is not clear. For example, surveillance at a large hospital where a commercial alcohol hand rub has been used for more than 10 years has not identified a single case of well-documented allergy to the product (606). In the few observed cases, however, it remains unclear whether the allergic reaction to the product is caused by the ethanol or by any of the auxiliary agents of the formulation (88). When reactions do occur, they may be caused by hypersensitivity to the alcohol itself, to aldehyde metabolites, or to some other additive (413). Allergic reactions to ethanol or isopropanol have been reported, are extremely rare (413), and depend on the chemical purity of the tested alcohol. Other ingredients in alcohol-based hand rubs that could be responsible for allergic reactions include fragrances, stearyl or isostearyl alcohol, benzyl alcohol, myristyl alcohol, phenoxyethanol, propylene glycol, parabens, and benzalkonium chloride (28, 107, 152, 189, 413, 442, 620).

In summary, ethanol (60 to 85%), isopropanol (60 to 80%) and *n*-propanol (60 to 80%) have very good activity against vegetative bacteria, mycobacteria, yeasts, dermatophytes, and enveloped viruses. Ethanol is more effective against naked viruses than are isopropanol and *n*-propanol. None of the alcohols has a potential for acquired bacterial resistance. Hand disinfection with an alcohol-based hand rub can reduce transient bacteria by 2.6 to 6.8 $\log_{10}$ units, but the effect on the resident hand flora is lower, with a mean reduction between 1.5 and 2.9 $\log_{10}$ units. The dermal tolerance is good (Table 9).

EFFECT ON NOSOCOMIAL INFECTIONS

Plain Soap (Social Hand Wash)

Compared with no hand washing at all, a simple hand wash reduces the transmission of nosocomial pathogens. Enforcement of a simple hand wash together with other infection control measures on a neonatal ICU led to a significant reduction of rectal colonization with VRE among newborns (40.2 versus 7%) (340). The simple hand wash has also been shown to be effective after direct contact with contaminated objects and before meals for prevention of infectious enteritis caused by *Salmonella enterica* servar Enteritidis (149). A similar effect on the incidence of diarrhea has been reported from India, although the hand wash had no effect on diarrhea caused by rotaviruses (512). One study exists which shows an effect even on the transmission of respiratory tract viruses. More frequent hand washing by health care workers in combination with cohorting of patients with respiratory tract infections caused by RSV has been found to reduce the nosocomial spread of RSV (225). Although these studies indicate that hand washing can reduce the transmission of nosocomial pathogens, especially during outbreak investigations involving multiple control measures, it is impossible to determine the individual contribution made by hand hygiene in preventing transmission.

Overall, wet hands have been described to significantly increase the risk of cross-transmission, indicating that hands should always be thoroughly dried (373).

Chlorhexidine and Triclosan (Hygienic Hand Wash)

Only a few studies were available which examined the impact of antimicrobial soaps on NIs. One study looked at the colonization and infection rate with *Klebsiella* spp. in an ICU. The annual rate was reduced from 22% in 1972 and 22.6% in 1973 to 15.5% in 1974, which was explained mainly by the introduction of a chlorhexidine-based liquid soap (81). In another study, NIs were less frequent when personnel performed antiseptic hand washing instead a simple hand wash (339). Antiseptic hand washing was also associated with lower NI rates in some ICUs but not in others (349). Some investigators have found that nosocomial acquisition of MRSA was reduced when the antimicrobial soap used for hand washing was changed (597, 621).

Ethanol, Isopropanol, and *n*-Propanol

The use of alcohol-based hand rubs in regular patient care and its promotion over the years resulted in an increase of compliance in hand hygiene from 48 to 66% and a decreased in the rate of NIs from 16.9 to 9.9% at the same time. This is

a significant decrease, of 41.1% in the NI rate (439). A comparative study of ICUs was carried out to determine the efficacy of a chlorhexidine-based soap (4%) and an isopropanol-based hand rub (60%) with the optional use of bland soap in reducing NIs. Washing hands with the chlorhexidine-based soap resulted in a lower rate of NIs, but the difference was not significant. However, it has been stated that this study does not indicate which of the two hand hygiene treatments is superior in ICUs. The personnel used much smaller volumes of isopropanol than of chlorhexidine and washed their hands more often than they used the hand rub (117). The data should therefore be regarded as resulting from a comparison between a social hand wash and chlorhexidine rather than a comparison between isopropanol and chlorhexidine (170). On a single ward in a 498-bed acute-care facility, use of an alcohol-based hand preparation over a 10-month period resulted in a 36% decrease in the incidence of two indicator NIs (urinary tract infections and surgical-site infections), expressed as the infection rate per 1,000 patient-days (211). In another study with an ethanol-based hand gel, the incidence of *C. difficile*-associated diarrhea decreased from 11.5 to 9.5 cases per 1,000 admissions within 1 year, but the difference was not significant (172). At the same time, the incidence of hospital-acquired MRSA decreased from 50 to 39% (172). Introduction of an ethanol-based hand disinfectant in a neonatal ICU significantly reduced cross-transmission of *K. pneumoniae* within 3 months from 21.5 to 4.7 cases of nosocomial colonization per 1,000 patient-days (75). Cross-transmission of *E. faecium* and *C. albicans* decreased as well, while rates for *E. coli*, *Enterobacter agglomerans*, and *E. faecalis* remained low and almost unchanged (75). The use of a virucidal hand rub based on 95% ethanol was part of an effective outbreak management of gastroenteritis caused by norovirus which involved 63 patients and health care workers (263).

EFFECT ON COMPLIANCE WITH HAND HYGIENE PRACTICES

Compliance with hand hygiene practices is known to be low. Compliance rates have been described to vary between 16 and 81% (437), with an overall average of 40% (71). One of the main goals of the new CDC guideline on hand hygiene is to provide evidence-based recommendations for improvement of compliance with hand hygiene (71). It is known that strategies to improve compliance with hand hygiene practices should be multimodal and multidisciplinary (435). Many individual parameters with a proven effect on hand hygiene compliance, however, have been identified in the new CDC guideline (71). These are efficacy, dermal tolerance, accessibility, time required for the procedure education, and personal perception; they are discussed below.

Hospital personnel should be provided with efficacious hand hygiene products, such as alcohol-based hand rubs. A change of the hand hygiene agent has been described to be particularly beneficial in institutions or hospital wards with a high workload and a high demand for hand hygiene (71).

Hand-washing agents are known to cause irritation and dryness, resulting in lower compliance rates (71). Hand hygiene products should have a low irritancy potential, particularly when these products are used multiple times per shift. A change to alcohol-based hand rubs should be made with great care, especially during winter, when hand skin is more irritable (252). Provision of skin care products may help (71, 437). However, they should not impair the efficacy of agents applied to the hands (71).

Easy access to a fast-acting hand hygiene agents should be viewed as the main tool of the strategy (71, 435, 437). Hand hygiene should be made possible, easy, and convenient. In areas with high workload, alcohol-based hand rubs should be made available at the entrance to the patient's room or at the bedside, in other convenient locations, or in individual pocket-sized bottles to be carried by health care workers (71).

Insufficient time to carry out the procedure, e.g., caused by high workload or understaffing, is associated with poor compliance (71). The time required for nurses to leave a patient's bedside, to to a sink, and wash and dry their hands before attending the next patient is a deterrent to a high compliance rate (71). A hand wash may take 62 s, whereas only one-fourth that time is required to use an alcohol-based hand rub placed at the bedside (579).

Ongoing education and promotion of hand hygiene should accompany the introduction of alcohol-based hand rubs in order to achieve long-lasting improvement in hand hygiene practices. Educational elements should include topics such as the rationale for hand hygiene, indications for hand hygiene, techniques of hand hygiene, methods to maintain hand skin health, and the correct use of gloves (71).

The smell, consistency ("feel"), and color may be important characteristics of the hand hygiene preparation that can influence the compliance rate by affecting the personal perception of those who use it (71). Differences in the acceptability of various agents have been described (258, 279, 349).

As well as the above parameters, the choice of the agent and the contents of a preparation may well have an impact on the compliance rate (242, 349, 481). The choice of a hand hygiene agent has been described to be one of many factors contributing to a strategy to successfully promote hand hygiene in hospitals (436). Baseline compliance rates in different departments vary between 30 and 63% for any soap, including plain and medicated soap, and sometimes even with the optional use of alcohol-based hand rubs if no intervention is done (Table 10). Several other studies have been conducted to measure the effect of various interventions on compliance rates with hand hygiene practices. In many studies the agent for hand hygiene remained unchanged. A higher compliance rate could be achieved by educational and training. In other studies, introduction of an alcohol-based hand rub or gel was accompanied by an educational and motivational campaign. Compliance rates could also be increased, often to a higher rate compared with the rate associated with no change of the hand hygiene agent (Table 11). Introduction of ethanol-based hand rubs sometimes revealed lower compliance rates and sometimes revealed higher compliance rates, with a trend toward the higher rates. The acceptability of the preparation and the role model function of physicians apparently have considerable influence. Preparations based on isopropanol or a combination of isopropanol and *n*-propanol revealed consistently higher compliance rates if education and promotion are carried out during introduction of the preparation and if the preparation has a superior dermal tolerance (Table 11). A 25% increase of

compliance with hand hygiene is possible (64) with the right choice of agent (which should have an excellent dermal tolerance and a high acceptability among users) and with an intensive educational and promotional campaign. These data very much support the recommendation of the CDC guideline to choose hand hygiene products with a low irritance potential and with a maximum acceptability by health care workers (71). The acceptability includes an assessment of the feel, fragrance, and subjective skin tolerance of the product (71). In this respect, well-formulated preparations based on propanol have been shown to have a better acceptability in terms of skin tolerance and skin dryness (279).

CONCLUSION

The social hand wash has only a few indications in hospitals and community medicine (243): mechanical cleaning when hands are visibly soiled with blood or other body fluids, before eating and after using the restroom, and if contamination of hands with bacterial spores is suspected (71). In these clinical situations, the simple hand wash reveals the best results compared with other possible hand treatments.

In the CDC guideline, a hygienic hand disinfection with an alcohol-based hand rub is the preferred treatment to be carried out after patient care activities that could lead to contamination of the hands of the health care workers, e.g., after contact with the patient's intact skin, body fluids or excretions, mucous membranes, nonintact skin, and wound dressings (if hands are not visibly soiled), when moving from a contaminated body site to a clean body site, after contact with environmental surfaces in the immediate vicinity of patients, and after glove removal (71). Hands should also be treated before having direct contact with patients, before donning sterile gloves when inserting devices such as vascular lines, indwelling urinary catheters, or peripheral vascular catheters (71). Hand hygiene is also indicated after using the restroom in cases of diarrhea and after blowing the nose in cases of an upper respiratory tract infection (18). The use of antimicrobial soaps in all these situations will probably be less effective in preventing cross-transmission of nosocomial pathogens and also has the risk of inducing occupational ICD.

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Exhibit C

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Evaluation of Virucidal Compounds for Inactivation of Rhinovirus on Hands

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Antimicrobial liquids and foams were applied to the hands to determine their virucidal activity against rhinoviruses. Dilute solutions (1%) of iodine in ethyl alcohol or in water were most effective in causing inactivation of rhinovirus when applied immediately after viral contamination. Iodine also had a residual virucidal activity which persisted on the hands for up to 1 h. Less effective inactivation of rhinovirus was observed with foams containing 0.23% hexachlorophene and 58% ethyl alcohol and another containing 0.2% benzalkonium chloride and 50% ethyl alcohol. Ethyl alcohol alone or in a mixture with benzyl alcohol was the least effective preparation tested.

Persons with rhinovirus colds contaminate their hands and objects in their environment with nasal secretions containing the infecting virus (1, 6, 8). Under experimental conditions, susceptible volunteers acquire virus on the fingers from contact with contaminated hands and, in turn, infect themselves by self-inoculation of the nose or eye (5). If this sequence of events is important in the natural spread of rhinoviruses (4), it might be possible to develop means of interrupting viral spread by the hand contact/self-inoculation route. One approach would be to apply virucidal compounds to the hands of infected individuals and/or their contacts. Although mechanical removal of virus from the hands can be achieved by washing with soap and water, this is not always practical. Also, hand washing cannot be tested in double-blind experiments designed to determine if hand contact/self-inoculation is an important natural route of rhinovirus spread.

The purpose of the current investigation was to evaluate compounds which, when applied to the hands in small amounts, might inactivate virus on the skin surface. A method was developed to artificially contaminate hands and then measure viral inactivation after treatment with virucidal liquids and foams. Compounds which were found to be effective under these conditions will later be tested for their ability to interrupt spread of experimental and natural infection.

MATERIALS AND METHODS

Virus. The rhinoviruses used for hand contamination were laboratory strains of types 29 and 39 grown in human embryonic lung cell (WI-38) cultures and stored in multiple portions at −70°C. A 1:10 dilution of stock virus in Hanks balanced salt solution (HBSS) served as the hand contaminant in all experiments.

Hand preparations. The preparations selected for testing are shown in Table 1. A volume of 1.0 ml of a liquid or a 3-cm-diameter sphere of foam was applied to each hand in all experiments.

Viral contamination followed by application of virucidal preparation. Before each experiment, the hands of a volunteer were washed thoroughly with soap and water and then dried with paper towels. When the volunteer's hands were used for multiple experiments, washing was done between experiments. The fingertips of both hands were then contaminated with 0.3 ml of virus. Virus was spread over the fingers by gentle rubbing. After the fingertips had air-dried, compounds were applied, vigorously rubbed over the hand, and allowed to dry completely. The fingertips of each hand, up to approximately the second joint, were then separately rinsed into a sterile petri dish with a total of 2.5 ml of beef heart infusion broth with 1% bovine serum albumin per hand. A 1-ml amount of the rinse was recovered and added to 1.0 ml of collecting broth with antibiotics. An infectivity titration of a 0.1-ml sample of the rinse was performed in WI-38 cell culture tubes. As a control, the viral contaminated hands of the same volunteer were sampled after no treatment and/or after treatment with 1 ml of water per hand.

Application of virucidal preparation followed by viral contamination. Following the demonstration of an effective immediate virucidal action of iodine solutions on skin contaminated with rhinovirus, studies were done to evaluate the residual virucidal activity of these compounds. The test preparation was applied first, and after varying intervals of time, the hands were contaminated with virus and then sampled. The methods of sampling and culture were the same as those described above except that after the 0.1-ml sample of the hand rinse was obtained for an infectiv-

ity titration, the remainder of the 2.0 ml of the undiluted rinse was inoculated equally into four WI-38 cell culture tubes.

Procedure for removal of toxicity from specimens. The liquid hand preparations tested in the experiments exhibited no toxicity to the cell cultures when samples of hand rinses were directly inoculated into WI-38 cells. The foam preparations produced cytotoxicity and, therefore, a technique of high-speed centrifugation was used to recover virus from the hand rinse sample. A 1-ml amount of the hand rinse was diluted with 4.0 ml of HBSS and centrifuged at 150,000 × *g* for 3 h at 4°C. The supernatant was discarded, and the button was resuspended in 1.0 ml of collecting broth to reconstitute the original volume before centrifugation. Infectivity titrations were performed with this material. Control samples, using measured amounts of virus in collecting broth, showed no loss of infectivity with this procedure.

RESULTS

Viral contamination followed by application of virucidal preparations. The infectivity titer (50% tissue culture infective dose [$TCID_{50}$]) of the type 39 rhinovirus pool used to contaminate the hands varied between $10^{5.5}$ and $\geq 10^{6.5}$/ml. When fingertips of the contaminated hands were cultured after no treatment, the titers of recovered virus ranged from $10^{2.0}$ to $10^{4.5}$ $TCID_{50}$/ml (geometric mean titer [GMT], $10^{3.3}$) (Table 2). After treatment with 1 ml of water, viral titers ranged from $10^{1.5}$ to $10^{4.0}$ $TCID_{50}$/ml. The GMT after water treatment was $10^{2.5}$ $TCID_{50}$/ml.

A total of 19 tests were conducted with 70% ethyl alcohol. Virus was not eradicated in any of these experiments. The GMT was $10^{1.8}$ $TCID_{50}$/ml, a $10^{0.7}$ reduction from that measured with water treatment. In an additional 16 experiments, contaminated hands were rubbed with a paper towel soaked in 70% ethyl alcohol. Infectious virus was not eliminated from the fingertips by this treatment; however, the GMT of the residual virus was reduced to $10^{1.5}$ $TCID_{50}$/ml. Three other liquid preparations were tested for activity against rhinovirus type 39. Virus was undetected in 1 of 16 fingertip rinses when a mixture of 79% ethyl alcohol, 10% isopropyl alcohol, and 0.1% tetrabromo-*o*-cresol was employed, resulting in a GMT of $10^{1.3}$ $TCID_{50}$ of residual virus per ml. With a mixture of 3% benzyl alcohol and 62% ethyl alcohol, virus was not detected in 2 of 12 rinses, and the GMT of residual virus was $10^{1.3}$ $TCID_{50}$/ml. The placebo (10% ethyl alcohol) did not eradicate virus in any of 12 tests and had a residual GMT of $10^{2.2}$ $TCID_{50}$/ml, which was similar to that of water treatment.

Two foam preparations were tested. With a mixture of 0.23% hexachlorophene and 58% ethyl alcohol virus was not detectable in 21 of 42 tests. In 11 tests $>10^{1.0}$ $TCID_{50}$ of virus per ml was recovered. The GMT of residual virus was $10^{0.6}$ $TCID_{50}$/ml. Virus was not detected in 9 of 16 tests with a mixture of 0.2% benzalkonium chloride and 50% ethyl alcohol. Residual virus was recovered in a titer of $>10^{1.0}$ $TCID_{50}$/ml in four tests. The GMT of residual virus was $10^{0.8}$ $TCID_{50}$/ml.

Similar experiments were conducted with hands contaminated with rhinovirus type 29 (Table 3). With no treatment of the hands, type 29 rhinovirus was recovered in 8 of 10 tests. The GMT of the positive specimens was $10^{2.1}$ $TCID_{50}$/ml. In 24 of 28 tests with water treatment, the titer of residual virus was $>10^{1.0}$ $TCID_{50}$/ml. The GMT of the residual virus was $10^{2.1}$ $TCID_{50}$/ml.

Four active and two placebo liquids were tested. Virus could not be recovered from 3 of 14 fingertip rinses of hands treated with a mixture of 6% benzyl alcohol and 62% ethyl alcohol. The GMT of residual virus was $10^{1.4}$ $TCID_{50}$/ml. The placebo (10% ethyl alcohol) did not eradicate virus from any of 14 hands; the GMT of residual virus was $10^{2.3}$ $TCID_{50}$/ml. Virus was not detected in 1 of 10 rinses when an iodophor (poloxamer-iodine complex) was used; the GMT of residual virus was $10^{1.7}$ $TCID_{50}$/ml. A 1% aqueous iodine solution was very effective in eliminating infectious rhinovirus from fingertip rinses. Virus was undetectable in 16 of 18 tests, and only low concentrations of virus were measured in the two positive rinses. Residual virus was detectable in all 12 tests with the iodine placebo. The GMT of the residual virus was $10^{1.6}$ $TCID_{50}$/ml.

TABLE 1. *Active ingredients of preparations tested for virucidal activity against rhinoviruses*

Liquids
70% Ethyl alcohol
70% Ethyl alcohol (in paper towels)
79% Ethyl alcohol, 10% isopropyl alcohol
3% Benzyl alcohol, 62% ethyl alcohol
6% Benzyl alcohol, 62% ethyl alcohol
10% Ethyl alcohol (placebo)
Poloxamer-iodine complex containing 1% available iodine
1% Iodine, 2% potassium iodide
0.01% Iodine, 0.02% potassium iodide, (placebo)
Foams
0.23% Hexachlorophene by weight, 58% ethyl alcohol by volume
0.2% Benzalkonium chloride, 50% (wt/wt) ethyl alcohol
10% (wt/wt) ethyl alcohol (placebo)

TABLE 2. *Comparison of virucidal activity of selected preparations on hands contaminated with rhinovirus type 39*

Prepn	No. of tests	Viral titer[a] ($TCID_{50}$/ml) in rinse from fingertips									GMT[c]
		<1.0	1.0[b]	1.5	2.0	2.5	3.0	3.5	4.0	4.5	
Control											
No treatment	24	0			1		7	7	8	1	3.3
Water	40	0		2	8	10	13	6	1		2.5
Liquid											
70% Ethyl alcohol	19	0	3	7	3	4	1	1			1.8
70% Ethyl alcohol (in towels)	16	0	4	5	5	2					1.5
79% Ethyl alcohol, 10% isopropyl alcohol	16	1	5	4	4	2					1.3
3% Benzyl alcohol, 62% ethyl alcohol	12	2		7	2		1				1.3
10% Ethyl alcohol (placebo)	12	0		3	3	3	3				2.2
Foam											
0.23% Hexachlorophene by weight, 58% ethyl alcohol by volume	42	21	10	9	2						0.6
0.2% Benzalkonium chloride, 50% (wt/wt) ethyl alcohol	16	9	3		2	2					0.8

[a] Log base 10.
[b] Virus was detected in one of two tubes inoculated with this dilution.
[c] Log_{10} $TCID_{50}$/ml. GMT, Antilog of sum total of samples × their respective log titers/total of all samples.

TABLE 3. *Comparison of virucidal activity of preparations on hands contaminated with rhinovirus type 29*

Prepn	No. of tests	Viral titer[a] ($TCID_{50}$/ml) in rinse from fingertips							GMT[c]
		<1.0	1.0[b]	1.5	2.0	2.5	3.0	3.5	
Control									
No treatment	10	2		3		1		4	2.1
Water	28	0	4	7	5	6	4	2	2.1
Liquid									
6% Benzyl alcohol, 62% ethyl alcohol	14	3	3	2	1	3	1	1	1.4
10% Ethyl alcohol (placebo)	14	0		1	3	6	3	1	2.3
Poloxamer-iodine complex	10	1		4	3	1		1	1.7
Iodine (aqueous)	18	16	1	1					0.25
Iodine (placebo)	12	0	2	6	3		1		1.5
Foam									
0.2% Benzalkonium chloride, 50% ethyl alcohol	6	6							0.25
10% Ethyl alcohol (placebo)	6	0	1	3	1	1			1.5

[a] Log base 10.
[b] Virus was detected in one of two tubes inoculated with this dilution.
[c] Log_{10} $TCID_{50}$/ml.

No measurable virus was obtained from six fingertip rinses when a foam containing 0.2% benzalkonium chloride and 50% ethyl alcohol was employed. Residual type 29 virus was present (GMT $10^{1.5}$ $TCID_{50}$/ml) in six rinses from hands receiving placebo foam.

Residual virucidal activity of aqueous iodine solution. The iodine solution was tested for residual virucidal activity against rhinovirus type 29 at 0.25, 0.5, and 1 h (Table 4). In 14 of 16 tests with water treatment, the residual viral titer was $>10^{1.0}$ $TCID_{50}$/ml, and the GMT varied from 1.4 to 2.1. In contrast, with iodine treatment, a residual titer of $>10^{1.0}$ $TCID_{50}$/ml was

TABLE 4. *Residual virucidal activity of aqueous iodine solution on hands contaminated with rhinovirus type 29*

Time interval (h)	No. of tests	Viral titers[a] ($TCID_{50}$/ml) in rinse from fingertips							GMT[c]
		<0.5[b]	0.5[b]	1.0	1.5	2.0	2.5	3.0	
Water									
0.25	6				5	1			1.4
0.5	6			1	3	2			1.4
1.0	4			1		1	1	1	2.1
Iodine									
0.25	18	12	3	3					0.2
0.5	18	12	4	2					0.2
1.0	8	2	1	4	1				0.6

[a] Log base 10.

[b] At these dilutions, four tubes each were inoculated with 0.5 ml of undiluted rinse.

[c] Log_{10} $TCID_{50}$/ml.

measured in only 1 of 44 tests and the GMT ranged from 0.2 to 0.6 for the three time intervals.

DISCUSSION

Klein and Deforest have established a classification in which viruses are placed into three different categories based on their susceptibility to chemical germicides (7). One group, the lipophilic viruses, have an outer envelope containing lipid, whereas a second group, although they do not contain a lipid envelope, can be inactivated by lipophilic virucides in the same manner as the lipophilic group. The picornavirus family, of which rhinoviruses are members, fall in the category of hydrophilic or nonenveloped viruses, possessing an inner core of nucleic acid and an outer protein coat. This third group of viruses has a strong resistance to lipophilic compounds and is, thus, resistant to many commonly used germicides.

In this investigation, selected virucidal compounds were evaluated by a method developed for testing preparations which were applied to the hands. The ideal compound for this use would be safe and nonirritative to skin after multiple applications and would have both immediate and prolonged residual virucidal activity, thus circumventing the need for mechanical removal of virus by frequent washing.

One percent iodine was the most effective of the compounds tested, showing both immediate and residual activity against type 29 rhinovirus. Virucidal activity of iodine has been recognized (2, 3), and its effectiveness against rhinovirus, when applied to the skin, is not surprising. The practicality of using hand preparations containing iodine is questionable, however, because of skin staining and the occasional occurrence of hypersensitivity (2, 3, 9). The iodine solutions can be used to conduct double-blind experiments in families to evaluate the importance of hand contact spread of rhinovirus. A placebo prepared from 2% food coloring and 0.01% iodine plus 0.02% potassium iodide had no virucidal activity and was indistinguishable from the active solution in color, skin staining, and odor. The staining property of iodine solutions, while a disadvantage for their ultimate practical use, does provide a way of assessing compliance when tested under field conditions.

Of the other preparations tested, only combinations of hexachlorophene and ethyl alcohol in foam and benzalkonium chloride and ethyl alcohol in foam showed substantial inactivation of rhinovirus on the hands. While ethyl alcohol alone is known to have some activity against hydrophilic or nonenveloped viruses (7, 11), its effect against rhinovirus, as shown in the current study, is limited. Hexachlorophene is insoluble in water (10), and its effect against hydrophilic viruses when used alone is unknown. Benzalkonium chloride is a markedly lipophilic compound which is ineffective against hydrophilic viruses (7). The reason for the relatively good rhinovirucidal action of foams containing either of these two compounds combined with ethyl alcohol is unknown. There is the possibility that a synergistic virucidal effect was produced by the combination of the compounds. Although a remote possibility, the mechanical effect of the breaking foam may have enhanced the virucidal activity of the ethyl alcohol.

Up to the present time, little work has been done to evaluate virucidal activity of compounds on the hands. If future epidemiological studies indicate that hand contact/self-inoculation is an important way in which rhinovirus colds spread, virucidal hand preparations should receive further investigation as a way of controlling these infections. The amount of rhinovirus involved in transmission of infection may not be large (5), and application of compounds with even moderate virucidal activity to the hands may be sufficient to interrupt the chain of transmission at this point. Safe and effective hand preparations can probably be developed for this use.

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Exhibit D

Submitted as Exhibit to Amendment of January 28, 2009

Rotavirus (cause of Severe Diarrhea)

About Rotavirus

Esta página en Español (about_rotavirus_spanish.htm)

On This Page:

Clinical features

Rotavirus is the most common cause of severe diarrhea among children, resulting in the hospitalization of approximately 55,000 children each year in the United States and the death of over 600,000 children annually worldwide. The incubation period for rotavirus disease is approximately 2 days. The disease is characterized by vomiting and watery diarrhea for 3 - 8 days, and fever and abdominal pain occur frequently. Immunity after infection is incomplete, but repeat infections tend to be less severe than the original infection.

The virus

A rotavirus has a characteristic wheel-like appearance when viewed by electron microscopy (the name rotavirus is derived from the Latin rota, meaning "wheel"). Rotaviruses are nonenveloped, double-shelled viruses. The genome is composed of 11 segments of double-stranded RNA, which code for six structural and five nonstructural proteins. The virus is stable in the environment.

Epidemiologic features

The primary mode of transmission is fecal-oral, although some have reported low titers of virus in respiratory tract secretions and other body fluids. Because the virus is stable in the environment, transmission can occur through ingestion of contaminated water or food and contact with contaminated surfaces. In the United States and other countries with a temperate climate, the disease has a winter seasonal pattern, with annual epidemics occurring from November to April. The highest rates of illness occur among infants and young children, and most children in the United States are infected by 2 years of age. Adults can also be infected, though disease tends to be mild.

Diagnosis

Diagnosis may be made by rapid antigen detection of rotavirus in stool specimens. Strains may be further characterized by enzyme immunoassay or reverse transcriptase polymerase chain reaction, but such testing is not commonly done.

Treatment

For persons with healthy immune systems, rotavirus gastroenteritis is a self-limited illness, lasting for only a few days. Treatment is nonspecific and consists of oral rehydration therapy to prevent dehydration. About one in 40 children with rotavirus gastroenteritis will require hospitalization for intravenous fluids.

Prevention

FDA News (http://www.fda.gov/bbs/topics/news/2006/NEW01307.html) - In a report issued February 3rd, 2006, the U.S. Food and Drug Administration approved a live, oral vaccine (RotaTeq™) for use in children. The Advisory Committee on Immunization Practices (ACIP) voted to recommend a newly licensed vaccine to protect against rotavirus.

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Exhibit E

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Coronavirus Particle Assembly: Primary Structure Requirements of the Membrane Protein

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Coronavirus-like particles morphologically similar to normal virions are assembled when genes encoding the viral membrane proteins M and E are coexpressed in eukaryotic cells. Using this envelope assembly assay, we have studied the primary sequence requirements for particle formation of the mouse hepatitis virus (MHV) M protein, the major protein of the coronavirion membrane. Our results show that each of the different domains of the protein is important. Mutations (deletions, insertions, point mutations) in the luminal domain, the transmembrane domains, the amphiphilic domain, or the carboxy-terminal domain had effects on the assembly of M into enveloped particles. Strikingly, the extreme carboxy-terminal residue is crucial. Deletion of this single residue abolished particle assembly almost completely; most substitutions were strongly inhibitory. Site-directed mutations in the carboxy terminus of M were also incorporated into the MHV genome by targeted recombination. The results supported a critical role for this domain of M in viral assembly, although the M carboxy terminus was more tolerant of alteration in the complete virion than in virus-like particles, likely because of the stabilization of virions by additional intermolecular interactions. Interestingly, glycosylation of M appeared not essential for assembly. Mutations in the luminal domain that abolished the normal O glycosylation of the protein or created an N-glycosylated form had no effect. Mutant M proteins unable to form virus-like particles were found to inhibit the budding of assembly-competent M in a concentration-dependent manner. However, assembly-competent M was able to rescue assembly-incompetent M when the latter was present in low amounts. These observations support the existence of interactions between M molecules that are thought to be the driving force in coronavirus envelope assembly.

Enveloped viruses acquire their lipid envelopes by budding through cellular membranes. The viral envelope contains integral membrane proteins that play important roles in envelope formation and virus entry. Three models have been proposed for the budding of enveloped viruses. Two of these are based on nucleocapsid-dependent assembly. In retroviruses the nucleocapsid is all that is required, since the Gag core particle can direct its envelopment without the need for viral envelope proteins (9, 23, 31). In contrast, both core and spike proteins are essential for the formation of togaviruses (26, 71), envelopment being driven by direct interactions between the viral envelope proteins and the nucleocapsid (42, 78). In the case of rhabdoviruses, optimal budding efficiency and virus production are achieved by a concerted action of spike and internal virus proteins (48). In the third model, budding is nucleocapsid independent. Coexpression of flavivirus prM and E results in the formation of subviral particles resembling capsidless viral envelopes (1, 44). The hepatitis B virus (HBV) surface proteins can be independently secreted from cells as subviral particles, which, however, are morphologically quite distinct from HBV virions (58, 67). Recently we demonstrated that coronavirus envelope proteins have the capacity to assemble uniform envelopes, which have the same appearance and dimensions as virus particles, independently of nucleocapsid proteins (73).

Coronaviruses are positive-stranded RNA viruses with 30-kb genomes packaged in helical nucleocapsids. The nucleocapsid is incorporated into a viral particle by budding into the intermediate compartment between the endoplasmic reticulum (ER) and the Golgi complex (33, 35, 72). The coronavirus membrane contains three or four viral proteins. The membrane (M) glycoprotein is the most abundant structural protein; it spans the membrane bilayer three times, leaving a short NH_2-terminal domain outside the virus (or exposed luminally in intracellular membranes) and a long COOH terminus (cytoplasmic domain) inside the virion (reviewed by Rottier [62]). The spike protein (S) is a type I membrane glycoprotein that constitutes the peplomers. The small envelope protein (E) has been detected as a minor structural component in avian infectious bronchitis virus (IBV), transmissible gastroenteritis virus (TGEV), and mouse hepatitis virus (MHV) particles (reviewed by Siddell [66]), but it has not been extensively characterized. Some coronaviruses also contain a hemagglutinin-esterase protein (HE) (reviewed by Brian et al. [7]).

Molecular interactions between the envelope proteins are thought to determine the formation and composition of the coronaviral membrane. M plays a predominant role in the intracellular formation of virus particles, for which S appears not to be required. Growth of coronaviruses in the presence of tunicamycin gave rise to the production of spikeless, noninfectious virions (27, 51, 63, 69). These particles were devoid of S but contained M. Independently synthesized MHV M protein accumulates in the Golgi complex (33, 34, 65) in homomultimeric complexes (36). Heterotypic interactions between the M and S proteins have been identified by coimmunoprecipitation and sedimentation analyses (55). The S protein, on its own, is transported to the cell surface, but when it is associated with the M protein, it is retained in the Golgi complex. Upon co-

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TABLE 1. Primers used in site-directed mutagenesis

No.	Sequence	Resulting mutant[a]
1	5′-GTCTAAACATACACGGTACCTTTC-3′	
2	5′-CCAAACATTATGAATAGTAC-3′	S_2N
3	5′-AAGGTACCAAACATTATGGCTGCTAC	A_2A_3
4	5′-AAGGTACCAAACATTATGAGTAGTGCTGC-3′	A_4A_5
5	5′-CAAACATTATGAGTAGTACAACACAAGCCGCAGAGG-3′	A_8A_{10}
6	5′-CCAAACATTATGGCTAGTAG	Ains2
7	5′-CCAAACATTATGCATCACCATCACCATCACAGTAGCACTACTCAGGCCC-3′	His
9	5′-CACAGAATTCTGATTGGATCC-3′	
10	5′-GTGTATAGATATGAAAGGTACCGTG-3′	
11	5′-GCTCTAGACTACAATGCTGTGTCCGCGCC-3′	Δ3
12	5′-GCTCTAGATCACAACAATGCGGTGTCCG-3′	Δ2
13	5′-GCTCTAGACTATCTCAACAATGCGGTGTC-3′	Δ1
14	5′-CGTCTAGATTAGATTCTCAACAATGCGG-3′	$T_{228}I$
15	5′-CGTCTAGATTAGATTCTCAACAATGCGG-3′	$T_{228}L$
16	5′-GCTCTAGATTAGACTCTCAACAATGCGG-3′	$T_{228}V$
17	5′-GCTCTAGATTAGTTTCTCAACAATGCGG-3′	$T_{228}N$
18	5′-GCTCTAGATTAGATATTATTTCTCAACAATGCGG-3′	OC
19	5′-CGTCTAGATCCGGTTCTCAACAATG-3′	+5
20	5′-GCTCTAGATTAGGTTGCCAACAATGCGGTG-3′	$R_{227}A$
21	5′-TTAGGTGTCCGCGCCACTCGGT-3′	Δ5
22	5′-CTATTTGTTTGAGGGCAGTCGG-3′	Δ11
23	5′-TTAATTTCCGACCTTGGACTTC-3′	Δ18
24	5′-GTCCAAGGTAGGAAACGGCCGACTGC-3′	$Y_{211}G$
PM149	5′-GATTACCATACACTAACA-3′	
LK-10	5′-CAACAATGCGGTGTCCGCGCCAC-3′	
LK-24	5′-CGCATTGTTGAGA(GATC)T(GC)TAATCTAAAC-3′	$T_{228}M$, $T_{228}I$, $T_{228}L$, $T_{228}F$, $T_{228}V$
LK-26	5′-GATATGAAGGGTACCATGTATGTTAGGCCG-3′	
LK-27	5′-CATCCTTAAAGTTTAGATTAGATATTATTTCTC-3′	BCV
LK-28	5′-TAATCTAAACTTTAAGGATGTCTTTTGTTC-3′	BCV
LK-29	5′-GCGATTATTTGGCCCACGGG-3′	
LK-30	5′-CACCGCATTGTTGAGA(AT)A(GT)TAATCTAAACTTTAAGG-3′	$T_{228}Y$
LK-31	5′-CACCGCATTGTTGAGAAATTAATCTAAACTTTAAGG-3′	$T_{228}N$
LK-32	5′-CACCGCATTGTTGAGATAGTAATCTAAACTTTAAGG-3′	Δ1
LK-38	5′-ACCGCATTGTTGTAGACCTAATCTAAAC-3′	Δ2
LK-39	5′-AGATTAGGTCTACAACAATGCGGTGTCCGCGCC-3′	Δ2
LK-40	5′-GACACCTAGTAGTTGAGAACCTAATCTAAAC-3′	Δ5
LK-41	5′-GGTTCTCAACTACTAGGTGTCCGCGCCACTCGG-3′	Δ5
LK-42	5′-GGAAATTAATAGCGACTGCCCTCAAACAAACCG-3′	Δ18
LK-43	5′-GGGCAGTCGCTATTAATTTCCGACCTTGGACTTC-3′	Δ18

[a] Primers 1 to 24 were used for construction of expression vectors for VLP mutants. Primers PM149 to LK-43 were used for construction of transcription vectors for the generation of viral mutants.

expression of M, S, and E by using the vaccinia virus T7 system (20), virus-like particles (VLPs) containing these three viral membrane proteins were assembled in and released from cells. However, only M and E were required for particle formation. The S protein was dispensable but was incorporated when present (6, 73). The envelope particles produced by this system were shown to form a homogeneous population of spherical particles indistinguishable from authentic virions in size and shape (73).

One of our main interests is to understand the process of coronavirus assembly. We are particularly interested in the interactions and the mechanisms that drive the formation of the viral particles. With the VLP assembly system, we have developed an ideal tool for the study of coronavirus envelope formation and for the analysis of the interactions between the viral membrane proteins in molecular detail. In the present study, we have used the VLP assembly system to investigate the primary structure requirements of the M protein in envelope formation. Site-directed mutations in the carboxy terminus of M were also incorporated into the MHV genome by targeted recombination. The results demonstrate that particle assembly is critically sensitive to changes in all domains of the M molecule.

MATERIALS AND METHODS

Cells and viruses. Recombinant vaccinia virus encoding the T7 RNA polymerase (vTF7-3) (20) was obtained from B. Moss. OST7-1 cells (15) (obtained from B. Moss) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 400 μg of G418/ml (all from Life Technologies, Ltd., Paisley, United Kingdom). Baby hamster kidney cells (BHK-21) obtained from the American Type Culture Collection (ATCC; Manassas, Va.) were maintained in the same medium lacking G418.

Expression vectors and transcription vectors. Expression construct pTM5ab contains the MHV open reading frames (ORFs) 5a and 5b, the latter encoding the E protein (73) in pTUG3 (74). Expression construct pTUMM contains the MHV strain A59 M gene (obtained from H. Niemann) cloned in the same vector as an *Xho*I fragment (73). The carboxy-terminal amino acid of the M protein coded by this clone is Thr (46, 54) rather than Ile, the terminal residue originally reported (2). A number of mutations were introduced into the M gene within this construct. Mutations in the amino-terminal domain (designated S_2N, A_2A_3, A_4A_5, A_8A_{10}, Ains2, and His) were made by PCR mutagenesis using 5′ terminal primers (Table 1, primers 2 through 7) directing the desired mutations and a 3′ internal primer (primer 1) corresponding to the region of the M gene that contains the unique *Kpn*I site. PCR fragments were first cloned into the pNOTA/T7 shuttle vector according to the Prime PCR Cloner procedure (5 Prime→3 Prime, Inc.) and were subsequently retrieved by cutting with *Kpn*I, after which the purified fragments were cloned into the expression vector pTUMM, from which the corresponding M fragment had been removed by using *Kpn*I. Mutants ΔN, ΔC and $G_{11}N_{13}$ were made by using single-stranded phagemid DNA according to the method of Zoller and Smith (79) as described previously (64). Mutant S_3N was made similarly by using primer 8. These mu-

tants were expressed from the transcription vector pTZ19R (47). For the construction of carboxy-terminal M mutants for VLP expression, an intermediate cloning vector was made as follows. By using a 5′ flanking primer (primer 9) and a 3′ terminal primer (primer 20), the M gene was prepared by PCR from a vector (pSFV1; Life Technologies, Inc.) containing the MHV M gene as a *Bam*HI fragment. The PCR fragment was cloned into the pNOTA/T7 shuttle vector. This vector was recleaved with *Bam*HI, and the resulting fragment was cloned into pBR322. The resulting cloning plasmid (pBMΔ5) contains the mutant M gene Δ5 as a *Bam*HI fragment and has a unique *Xba*I site flanking the 3′ terminus. The Δ3, Δ2, Δ1, $T_{228}I$, $T_{228}L$, $T_{228}V$, $T_{228}N$, OC, +5, and $R_{227}A$ mutant M genes were all made by PCR mutagenesis using a 5′ internal primer (primer 10) containing the unique *Kpn*I site and a 3′ terminal primer containing the desired mutation and an *Xba*I site (primers 11 through 20). The PCR fragments were digested with *Kpn*I and *Xba*I and cloned into pBMΔ5 that had been treated with the same enzymes. The mutant M genes were finally cleaved out with *Bam*HI and cloned into expression vector pTUG3. Mutants Δ18, Δ11, and Δ5 were also made by PCR mutagenesis using a 5′ internal primer (primer 10) and 3′ terminal primers (primers 21 through 23), and the PCR fragments were cloned into pGEM-T (Promega). The plasmids were digested with *Kpn*I and *Spe*I, and the resulting fragments were cloned into expression vector pTUMM treated with *Kpn*I and *Xba*I. The $Y_{211}G$ mutant was made with the Altered Sites site-directed mutagenesis kit purchased from Promega. The MHV M gene was cloned as a *Bam*HI fragment into pALTER-1. Primer 24 was used to introduce the mutations, and pALTER-1 was used as the expression vector.

For the construction of carboxy-terminal M mutants to be incorporated into the MHV genome, splicing overlap extension (SOE)-PCR was used as described previously (59) to create mutations in the transcription vector pCFS8 (16). This plasmid encodes a runoff transcript that contains a 5′ segment of the MHV genome fused to the entire 3′ end of the genome beginning with the S gene and is tagged with a 19-base substitution in gene 4 (16). PCR products containing the $T_{228}M$, $T_{228}I$, $T_{228}L$, $T_{228}F$, and $T_{228}V$ mutations were generated by two rounds of PCR using inside primers LK-24 (which is partially degenerate) and LK-10 with external primers PM149 (upstream) and LK-29 (downstream). The same scheme was used to produce the $T_{228}Y$, $T_{228}N$, and Δ1 mutations, substituting primers LK-30, LK-31, and LK-32, respectively, for LK-24. Similarly, PCR products containing the Δ2, Δ5, and Δ18 mutations were generated with the inside primer sets LK-38 and LK-39 (Δ2), LK-40 and LK-41 (Δ5), and LK-42 and LK-43 (Δ18). Each PCR product was restricted with *Bss*HII and *Bsr*FI and was incorporated into the parent vector via a three-way ligation with the *Bsr*FI-*Nhe*I and *Bss*HII-*Nhe*I fragments of pCFS8. The $T_{228}F$ mutant turned out to have a second, unintended mutation generating the substitution $T_{223}I$. For the construction of a chimeric mutant exchanging the carboxy-terminal half of the MHV M protein with that of bovine coronavirus (BCV), SOE-PCR was used to generate a perfect substitution bounded by the *Kpn*I site and the M-gene stop codon. In the first round of PCR, primers LK-26 and LK-27 were used to amplify the 3′ terminus of the BCV M gene from plasmid p(M+N)CAT1 (provided by David Brian), and primers LK-28 and LK-29 were used to amplify the downstream MHV region (the M-N intergenic junction and the 5′ end of the N gene) from pFV1 (16), which is identical to pCFS8 except that it does not contain the gene 4 tag. The second-round PCR product, obtained from primer pair LK-26 and LK-29 by using the first-round products as the template, was then restricted with *Kpn*I and *Bsr*FI and was ligated with the *Bsr*FI-*Nhe*I fragment of pCFS8 into an appropriate subclone. Finally, the fragment running from the *Eco*RV site at the end of gene 5 through the *Nhe*I site in the N gene was transferred from this intermediate to the vector pFV1. All PCR constructs were verified by sequencing.

Infection and transfection. Subconfluent monolayers of OST-7 and BHK-21 cells in 10-cm^2 tissue culture dishes were inoculated at 37°C with vTF7-3 in DMEM at a multiplicity of infection of 10. After 1 h (t = 1 h), cells were washed with DMEM and medium was replaced with transfection mixture, consisting of 0.2 ml of DMEM without FCS but containing 10 μl of Lipofectin (Life Technologies) and 5 μg of each selected construct. After 10 min at room temperature (RT), 0.8 ml of DMEM was added and incubation was continued at 37°C. At t = 2 h, cells were transferred to 32°C and incubation was continued.

Metabolic labeling and immunoprecipitation. At t = 4.5 h, cells were washed with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} (PBS^{++}) and were starved for 30 min in cysteine- and methionine-free MEM, containing 10 mM HEPES, pH 7.2, without FCS. The medium was then replaced by 600 μl of the same medium containing 100 μCi of ^{35}S in vitro cell labeling mix (Amersham). After 3 h, cells were placed on ice, and the media were collected and cleared by centrifugation for 15 min at 4,000 × g and 4°C. Cells were washed with ice-cold PBS^{++} and lysed with lysis buffer, consisting of 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholic acid (NaDOC), 0.1% sodium dodecyl sulfate (SDS), 2 μg of aprotinin/ml, 2 μg of leupeptin/ml, and 1 μg of pepstatin A/ml. Lysates were cleared by centrifugation for 10 min at 10,000 × g and 4°C. Radioimmunoprecipitation was performed on lysates diluted 5 times with immunoprecipitation buffer, consisting of 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1% NaDOC, 0.1% SDS, and protease inhibitors. Culture media were prepared for immunoprecipitation by addition of 1/4 volume of 5-times-concentrated lysis buffer. Rabbit anti-MHV serum k134 (61) was used at a 500-fold dilution for immunoprecipitation of MHV proteins at 4°C. The immune complexes were adsorbed to Pansorbin cells (Calbiochem) for 30 min at 4°C and were subsequently collected by centrifugation. Pellets were washed three times by resuspension and centrifugation using 20 mM Tris-HCl (pH 7.6)–150 mM NaCl–5 mM EDTA–0.1% NP-40 followed by a single wash using 20 mM Tris-HCl (pH 7.6)–0.1% NP-40. The final pellets were suspended in electrophoresis sample buffer and heated at 95°C for 2 min before analysis by SDS-polyacrylamide gel electrophoresis (PAGE) using a 15% polyacrylamide gel according to the method of Laemmli (38). In some cases immunoprecipitates were digested with endoglycosidase F/*N*-glycosidase F (glyco F; Boehringer Mannheim) as described earlier (10) before analysis by SDS-PAGE.

Indirect immunofluorescence. Indirect immunofluorescence was performed on BHK-21 cells grown on 12-mm coverslips. The morphology of these cells makes them more convenient than OST7-1 cells for this assay. Cells were fixed at t = 5 h, permeabilized, and stained for immunofluorescence as described previously (33). The rabbit anti-MHV serum k134 was used at a 1:400 dilution.

Construction of MHV mutants. Carboxy-terminal M mutations were incorporated into the genome of MHV by targeted recombination between synthetic donor RNA from *Hin*dIII-truncated transcription vectors and the thermolabile N-gene deletion mutant Alb4 as described previously (16, 45, 59). Candidate recombinant viruses were plaque purified and analyzed by reverse transcription-PCR (RT-PCR) and sequencing of RNA from infected cells. Final confirmation of the construction of individual mutants came from direct sequencing of RNA isolated from purified virions (16, 59).

RESULTS

Effects of cytoplasmic-domain mutations on VLP assembly. In order to elucidate the primary structure requirements of the MHV M cytoplasmic domain for virus assembly, we constructed a number of mutants. Mutant ΔC has a large internal deletion, removing residues E_{121} through D_{195}, which comprise most of the amphiphilic domain. Mutant Δ18 lacks the carboxy-terminal hydrophilic domain. Since this deletion appeared to have a drastic effect on VLP assembly, a series of mutants was made with progressively smaller deletions at the carboxy terminus, ranging from 11 amino acids to a single amino acid (Fig. 1). The abilities of these mutant M molecules to function in assembly were tested by coexpressing each of the mutant genes with the E protein gene. Genes were expressed by using the recombinant vaccinia virus bacteriophage T7 RNA polymerase system in OST7-1 cells. Proteins were labeled with ^{35}S-labeled amino acids from 5 to 8 h postinfection. Cells and media were collected separately and processed for immunoprecipitation with a polyclonal rabbit anti-MHV serum, followed by SDS-PAGE using a 15% polyacrylamide gel. Analysis of the cell lysates (Fig. 2) of the single expressions demonstrated that all mutant constructs were expressed and yielded products of expected sizes. In all cases M appeared as a set of proteins differing in apparent molecular size, due to different extents of O glycosylation (34, 72). The patterns of the glycosylated species of M mutants were not much different from that of the wild-type (WT) M protein, indicating that the mutations in the carboxy terminus did not affect the ability of the amino terminus to become glycosylated, nor did they affect the ability of the proteins to be transported to the Golgi complex, as the slowest-migrating forms of M are known to result from modifications occurring in this organelle (34). Transport to the Golgi complex was confirmed by immunofluorescence analyses as illustrated in Fig. 3. The mutant M proteins were also analyzed for their stability by pulse-chase experiments and appeared to be as stable as WT M. In the double expressions, the presence of the E protein did not seem to affect the synthesis of M qualitatively under the experimental conditions used; in some cases, the expression level of M was somewhat decreased. The E protein itself was not resolved due to poor recognition by the antiserum, but its synthesis was confirmed by using an E-specific serum (data not shown). Particle assembly and secretion were assayed by measuring the release of the M protein into the culture medium. The E protein was extremely difficult to detect in VLPs, due to its small size and very

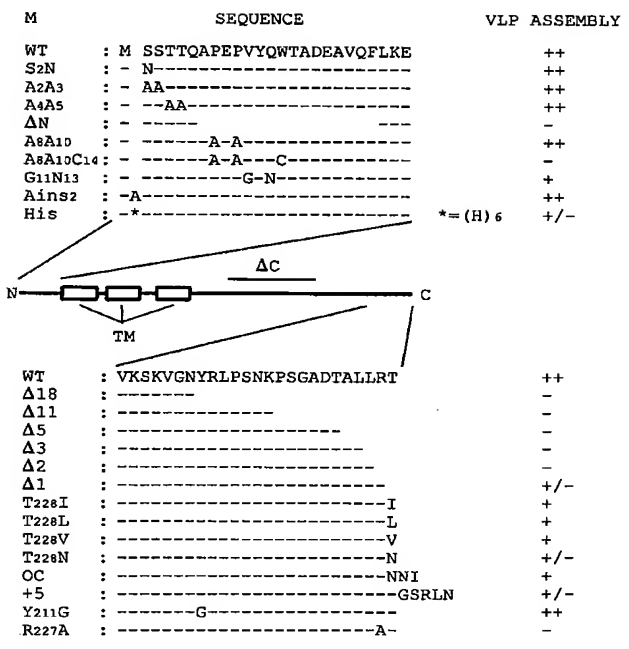

FIG. 1. Overview of mutant M proteins. In the middle is a schematic representation of the structure of the M protein, with the three transmembrane (TM) domains indicated. Amino acid sequences of the amino-terminal and carboxy-terminal domains, and of the mutants with mutations in these domains, are shown above and below the diagram, respectively. Dashes represent unchanged residues, gaps represent deletions. The domain deleted in mutant ΔC (residues E_{121} through D_{195}) is indicated by a horizontal line. The abilities of the different M proteins to support VLP assembly is indicated at the right. The ratio of the amount of M present in the culture medium to that in cells was taken as a measure for VLP assembly by using WT M as a reference. The semiquantitative scores ++, +, +/−, and − indicate efficient, moderately efficient, inefficient, and nonexistent VLP synthesis, respectively.

low abundance (73). It is clear from observation of WT M protein that no M was released into the medium unless the E protein was coexpressed, consistent with our earlier findings (73). However, all mutant M proteins failed to be secreted into the culture medium when they were expressed in combination with the E protein. Not only an internal deletion in the cytoplasmic domain but also deletions at the carboxy terminus were fatal for VLP assembly. Even the deletion of one single amino acid at the extreme carboxy terminus abolished VLP formation almost completely (Fig. 2), indicating that the carboxy-terminal residue is very important.

We therefore prepared a number of additional mutants with various changes at the very carboxy-terminal end. This second set of M mutants consisted of a panel of molecules in which the C-terminal threonine residue was replaced either by isoleucine (which is present at this position in a number of MHV strains [28]) or by leucine, valine, or asparagine. Furthermore, we prepared a mutant (OC) with a carboxy-terminal sequence identical to that of the human coronavirus (strain OC43) and BCV M proteins; a mutant with an extension of 5 foreign amino acids (+5); a mutant with a replacement of the Y at position 211 by G ($Y_{211}G$), which allows the protein to be detected at the cell surface (unpublished data); and a mutant in which R at position 227 was replaced by A ($R_{227}A$) (Fig. 1). These mutant constructs were expressed alone and in combination with the E protein gene. Cells and media were processed and analyzed as described above. The results (Fig. 4) showed that all mutants were expressed, producing proteins of the expected sizes. All M mutants again appeared as a set of differently glycosylated species not much different from that of WT M, indicating that they all had preserved the ability to become glycosylated and to be transported to the Golgi complex. When the appearance of the M protein in the culture medium is used as a measure for VLP assembly, it is clear that no M proteins were secreted during the single expressions. When the E protein was coexpressed, mutants $T_{228}I$, $T_{228}L$, $T_{228}V$, OC, and $Y_{211}G$ were released into the medium, although with decreased efficiency compared to WT. Mutants $T_{228}N$ and +5 almost completely failed to be secreted, while mutant $R_{227}A$ was not secreted at all. Quantitative analysis using a phosphorimager, taking the ratio of the amount of M present in the culture medium to that in cells as a measure of VLP assembly, showed that $T_{228}I$, $T_{228}L$, $T_{228}V$, OC, and $Y_{211}G$ had two- to fourfold reductions in VLP yield. For mutants $T_{228}N$ and +5, this decrease was 10- to 20-fold; the extension with 5 foreign amino acids was more detrimental for VLP assembly than replacement of the C-terminal residue by asparagine. These results demonstrate that VLP assembly is very sensitive to changes at the extreme C terminus. The tyrosine at position 211 does not seem to be important for VLP assembly (Fig. 4).

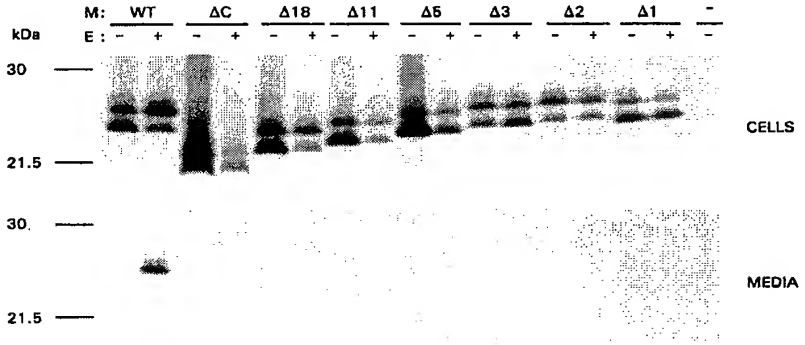

FIG. 2. Effects of deletions in the M cytoplasmic domain on VLP assembly. OST7-1 cells infected with recombinant vaccinia virus vTF7-3 were transfected with a plasmid containing the WT or mutant M gene either alone or in combination with a plasmid containing the E protein gene, each gene behind a T7 promoter. Cells were labeled for 3 h with ^{35}S-labeled amino acids. Both cells (upper panel) and the culture medium (lower panel) were prepared and used for immunoprecipitation, and the precipitates were analyzed by SDS-PAGE. The different M genes expressed are indicated above each set.

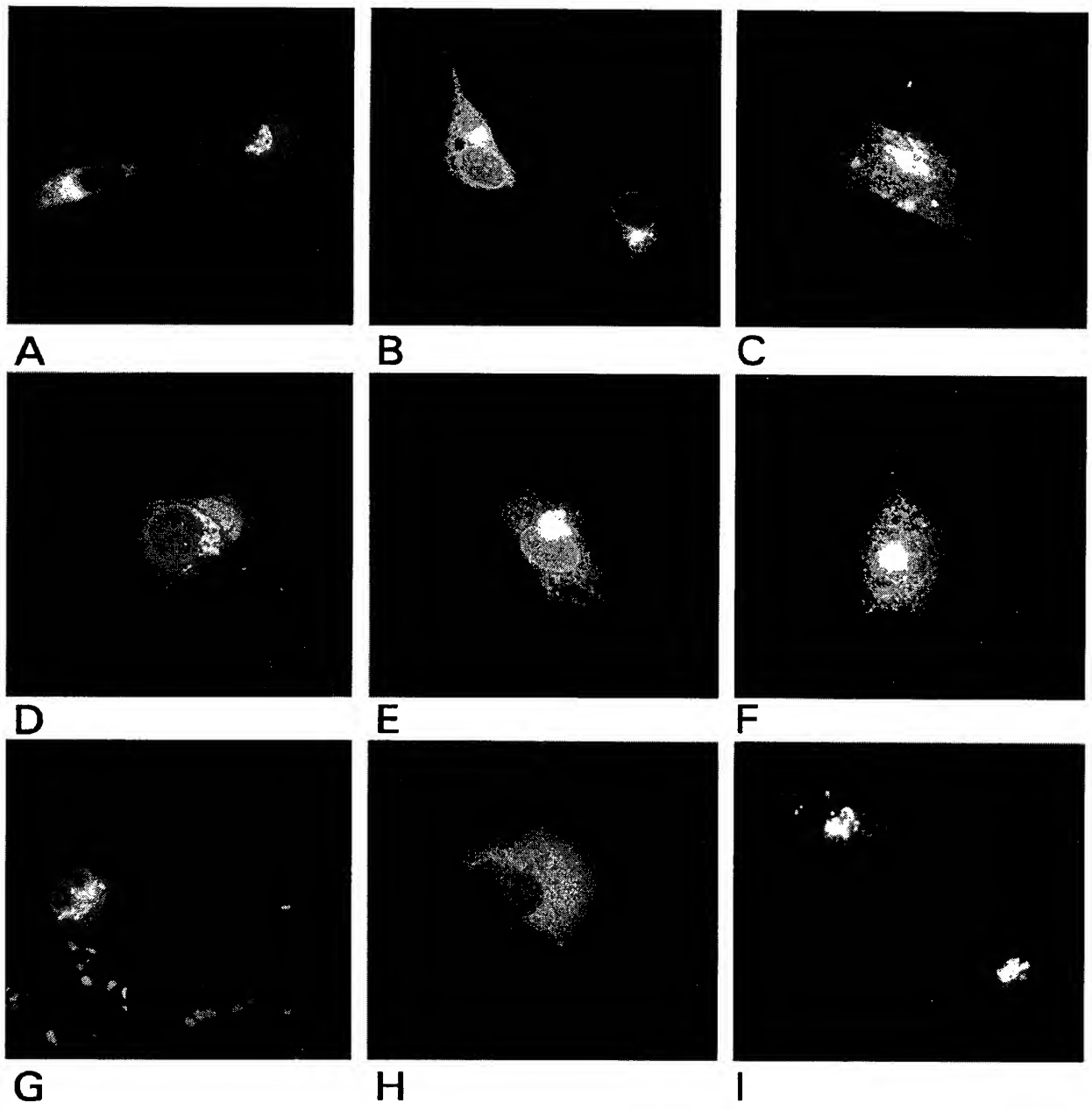

FIG. 3. Indirect immunofluorescence analysis of mutant M proteins. vTF7-3 infected BHK-21 cells were transfected with plasmids encoding WT M (A) or the ΔC (B), Δ18 (C), ΔN (D), A_4A_5 (E), A_8A_{10} (F), or $A_8A_{10}C_{14}$ (G) mutant or were mock transfected (H and I). Cells were fixed at 5 h postinfection and processed for immunofluorescence with the anti-MHV serum k134 (A through H) or a polyclonal rabbit serum against the resident Golgi protein α-mannosidase II (I) (a kind gift from K. Moremen [50]).

Effects of transmembrane deletions on VLP assembly. To assess the role of the transmembrane domains in VLP assembly, mutant proteins lacking either the first (Δa), the second (Δb), or the third (Δc) transmembrane domain or combinations thereof [Δ(a+b), Δ(b+c), and ΔaΔc] were tested for their VLP-forming abilities. The construction of these mutants and their expression in vitro and in vivo have been described earlier (37). When these mutant M proteins were coexpressed with E, no VLPs were detected in any case (data not shown). These results indicate the importance of the transmembrane domains in preserving the functional structure, orientation, and localization of the M molecule.

Effects of amino-terminal domain mutations on VLP assembly. Next, we wanted to investigate whether VLP formation is

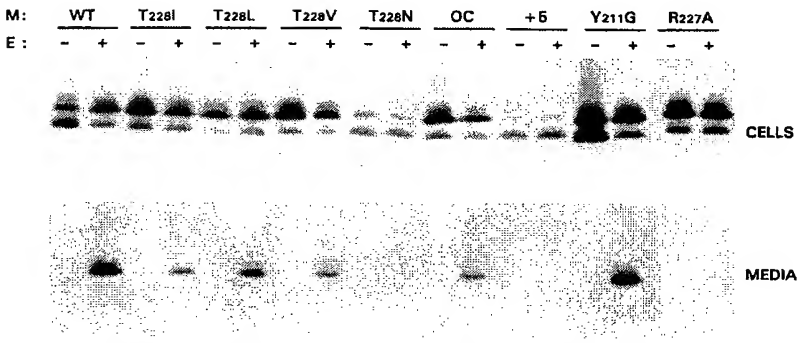

FIG. 4. Effects of mutations in the M carboxy-terminal domain on VLP assembly. Expression of M and E genes was performed as described in the legend to Fig. 2. The different M genes tested are indicated above the gels.

also sensitive to changes in the amino-terminal domain, i.e., the luminal domain of the M protein. For this purpose another set of mutants was constructed with various mutations in this domain. In mutant A_2A_3, the serines at positions 2 and 3 were replaced by alanines, while in mutant A_4A_5, the threonines at positions 4 and 5 were replaced by alanines (Fig. 1). Mutant ΔN lacks residues A_7 through F_{22}, resulting in an internal deletion of 16 amino acids. Three mutants have substitutions in this domain. In mutant A_8A_{10}, the prolines at positions 8 and 10 were replaced by alanines. $A_8A_{10}C_{14}$ also has a cysteine substitution for the tryptophan at position 14, which was fortuitously obtained during the construction of mutant A_8A_{10}. Mutant $G_{11}N_{13}$ has replacements of valine and glutamine at positions 11 and 13 with glycine and asparagine, respectively. Furthermore, two mutants with insertions between the initiating methionine and the serine at position 2 were constructed. Mutant Ains2 has an insertion of 1 alanine, while mutant His has a stretch of 6 histidines inserted for purification purposes. The mutant constructs were expressed alone and in combination with the E protein gene. Cells and media were processed and analyzed as described above. Analysis of the cell lysates (Fig. 5) demonstrated that in all cases mutant M proteins of expected sizes were expressed but that their glycosylation patterns were variously affected. It should be noted that O glycosylation of MHV M occurs at a threonine in the amino-terminal domain (unpublished data). Thus, mutant A_4A_5 did not become glycosylated, and this did not reflect an inability to be transported to the Golgi complex, as was verified by immunofluorescence (Fig. 3E). The same holds true for mutant ΔN; the deletion blocked glycosylation at the threonines without affecting intracellular transport (Fig. 3D). Transport was also unaffected for mutants A_8A_{10} and $A_8A_{10}C_{14}$ (Fig. 3F and G). The extents of glycosylation of these mutants were decreased, apparently due to the replacements of the prolines. The normal pattern of differently glycosylated species was observed with mutants A_2A_3, $G_{11}N_{13}$ and Ains2. The His mutant also showed the usual pattern of glycosylation, indicating that the insertion of the histidine stretch did not interfere with the membrane translocation of the amino-terminal domain, which occurs through the action of an internal signal sequence (61). The normal glycosylation also indicates that the protein's transport to the Golgi complex was not affected. One reason to prepare mutant $G_{11}N_{13}$ was to obtain an N-glycosylated form of MHV M. The N glycosylation consensus sequence generated by the introduction of the asparagine at position 13 appeared, however, not to be used by the cell. The protein's modifications are indistinguishable from those of WT M and are insensitive to endoglycosidases that remove N-linked sugars.

In considering VLP formation, it is clear from Fig. 5 that all mutant M proteins failed to be secreted into the culture medium when expressed alone. When coexpressed with the E protein, mutants A_2A_3 and A_4A_5 were secreted into the medium with efficiency similar to that of WT M. This result indicated that neither the serines nor the threonines are primary structure requirements for VLP formation. Interestingly, O glycosylation of the M protein is not a prerequisite for VLP assembly and release, since the unglycosylated mutant A_4A_5

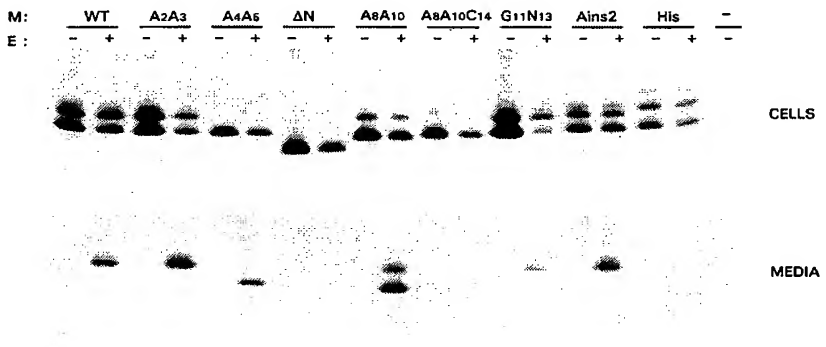

FIG. 5. Effects of mutations in the amino-terminal domain of M on VLP assembly. M and E genes were expressed as described in the legend to Fig. 2.

was found in the medium. Mutant ΔN was not secreted, indicating that the deleted part of the amino-terminal domain is important for VLP formation. Consistently, other mutations in this region also showed drastic effects: mutant $G_{11}N_{13}$ had reduced VLP-forming ability, while complete inhibition was observed with mutant $A_8A_{10}C_{14}$. Since mutant A_8A_{10} was secreted efficiently, the prolines do not seem to be important. The relatively high proportion of the unglycosylated form of the secreted protein reflects the decreased extent of glycosylation of this mutant. When mutant $A_8A_{10}C_{14}$ was analyzed under nonreducing conditions, it was resolved as a monomer (data not shown), indicating that the cysteine introduced at position 14 did not lead to formation of a disulfide bridge between M molecules. Mutant Ains2 was secreted into the medium efficiently; hence, it is clear that insertion of 1 alanine at position 2 does not affect the ability of the M protein to form VLPs. Insertion of the histidine stretch, however, strongly impaired VLP assembly, indicating that only minor insertions are allowed at this position.

Assembly of N-glycosylated M protein in VLPs. Coronavirus M proteins are either N glycosylated or O glycosylated in the amino-terminal domain (62). Murine coronaviruses belong to the latter category. Above we showed that O glycosylation is not required for MHV membrane assembly. In order to investigate whether MHV M would still be able to function in assembly as an N-glycosylated protein, we constructed a mutant (S_2N) in which the serine residue at position 2 was replaced by an asparagine, thus creating an N glycosylation consensus sequence (Asn-X-Ser/Thr [22]). The mutant construct was expressed alone or in combination with the E protein gene. Initial expression studies with the mutant showed that the protein can become both O and N glycosylated in a complex pattern that will be described elsewhere (unpublished data). One major complication was the maturation of the N-linked side chain by extensive heterogeneous modifications, resulting in a diffuse smear in the gel, which hampered the detection of N-glycosylated M protein in cells and VLPs. In order to avoid this problem, we used an inhibitor of oligosaccharide maturation, 1-deoxy-mannojirimycin (DMJ), which interferes with the action of α-mannosidase I, keeping the sugars in a simple, endoglycosidase H (endo H)-sensitive form (21). In other cases, cells were treated with tunicamycin, a general inhibitor of N glycosylation (reviewed by Elbein [14]). Cells and media were processed and analyzed as described above. Prior to gel electrophoresis, some immunoprecipitates were treated with glyco F to remove the N-linked sugars. Analysis of the cell lysates (Fig. 6) showed the mutant S_2N protein to appear both in an unglycosylated form (lower band; about 23 kDa) and as some N-glycosylated species (lanes 5 and 6). The distinct band of about 28 kDa consisted of M protein carrying N-linked sugars that were endo H sensitive (data not shown). The endo-H-resistant S_2N was differentially glycosylated and could not be distinguished from the background due to its heterogeneity. After treatment with glyco F, the N-linked sugars were removed, resulting in the typical pattern of differently O-glycosylated M species (Fig. 6, lanes 7 and 8). Hence, S_2N was both N and O glycosylated. After treatment with DMJ, the N-glycosylated M proteins appeared as a 28- and a 30-kDa species (Fig. 6, lanes 9 and 10). When immunoprecipitates were treated with glyco F, the typical pattern of differently O-glycosylated M species was again observed (Fig. 6, lanes 11 and 12). DMJ did not influence expression of WT M quantitatively or qualitatively (Fig. 6, lanes 3 and 4). Treatment of S_2N-expressing cells with tunicamycin resulted in S_2N that was O glycosylated but not N glycosylated (Fig. 6, lanes 13 and 14).

Analysis of the media (Fig. 6) showed that when the S_2N mutant was coexpressed with E in the absence of DMJ (lanes 5 to 8), little S_2N was detected in the medium. None of the unglycosylated form and hardly any of the 28-kDa N-glycosylated form appeared to be released. Only some heterogeneously glycosylated M protein was secreted (Fig. 6, lane 6), partly representing double-glycosylated material, as became apparent after glyco F treatment (lane 8). VLP release was much higher when oligosaccharide maturation was prevented by DMJ (Fig. 6, lanes 9 to 12). Significant amounts of the now immature, double-glycosylated form were secreted into the medium. Apparently, N glycosylation per se did not affect VLP assembly strongly. DMJ itself had a slight but distinct inhibitory effect on VLP formation or release, as was clear from the interference with production of WT-based particles (Fig. 6, lanes 2 and 4). When N glycosylation of S_2N was blocked by using tunicamycin, normal amounts of VLPs, containing the normal O-glycosylated forms of M, were produced (Fig. 6, lane 14). Apparently, the mutation itself did not interfere with VLP assembly.

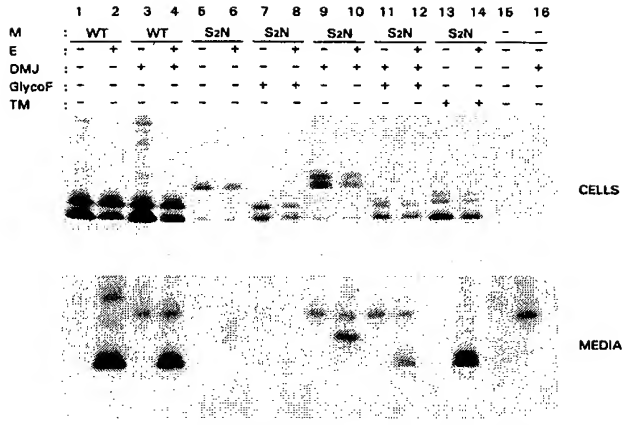

FIG. 6. Assembly of an N-glycosylated form of the MHV M protein into VLPs. OST7-1 cells infected with recombinant vaccinia virus vTF7-3 were transfected with a plasmid containing the WT or the S_2N mutant M gene, either alone or in combination with a plasmid containing the E protein gene, each gene behind a T7 promoter. Cells were labeled for 3 h with ^{35}S-labeled amino acids. In some cases, cells were treated with 1 mM DMJ (lanes 3 and 4, 9 to 12, and 16); in other cases, cells were treated with 5 μg of tunicamycin (TM)/ml (lanes 13 and 14). Both cells (upper panel) and the culture medium (lower panel) were prepared and used for immunoprecipitation, and the precipitates were analyzed by SDS-PAGE. Prior to gel electrophoresis, some immunoprecipitates were treated with glyco F to remove N-linked sugars (lanes 7, 8, 11, and 12).

Inhibition of normal VLP formation by M proteins with carboxy-terminal tail deletions. Interactions between M molecules are considered essential in coronavirus envelope assembly. It was therefore of interest to analyze whether and how mutant M proteins that are themselves deficient in VLP formation would interfere with the assembly of VLPs driven by WT M and E. Therefore, a triple-expression experiment in which these proteins were coexpressed with different carboxy-terminal tail mutants was performed. Fixed amounts of plasmid DNA encoding WT M and E were used in transfection, while an equal or a 5-times-lower amount of the plasmid DNAs specifying the mutant M proteins was used. Cells and media were processed as described above. Analysis of the cell lysates (Fig. 7) in all cases showed the differently glycosylated M species. Due to the small differences in size, mutants Δ5, Δ2, and Δ1 could not be discriminated from WT M. The unglycosylated form of mutant Δ18 could be distinguished, but its

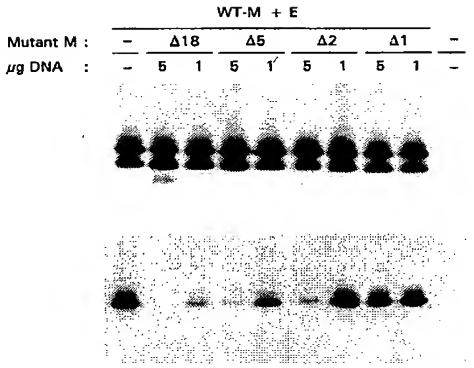

FIG. 7. Inhibition of VLP formation by mutant M proteins with carboxy-terminal deletions. After infection of OST7-1 cells with recombinant vaccinia virus vTF7-3, cells were transfected with plasmid DNA encoding WT M and E (5 μg of each) together with 1 or 5 μg of plasmid DNA encoding mutant M. The different M mutants expressed are indicated above the gels. Cells were labeled for 3 h with ^{35}S-labeled amino acids. Both cells (upper panel) and the culture medium (lower panel) were prepared and used for immunoprecipitation, and the precipitates were analyzed by SDS-PAGE.

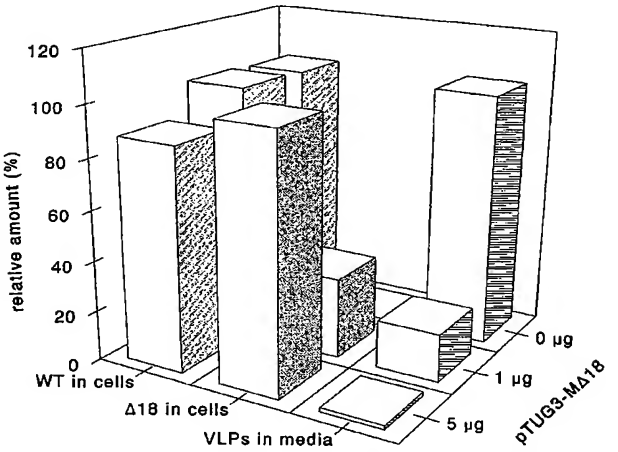

FIG. 8. Quantitation of the concentration-dependent inhibition of VLP formation by mutant M protein Δ18. The relative amounts of glycosylated WT or unglycosylated Δ18 mutant M protein present in cells and media for each transfection condition shown in Fig. 6 were determined by using a phosphorimager. The relative amount of WT M secreted (i.e., VLPs in media) is also shown.

glycosylated forms were also obscured by WT M. In this case the glycosylated species of WT M could be discriminated from those of the mutant because the former run slightly slower. The results indicate that the expression of WT M was hardly affected by the coexpression of mutant Δ18, even when equal amounts of their plasmids had been cotransfected. Judging from the amounts of the unglycosylated forms, it seems that the efficiency of expression was higher for WT M than for the mutant.

Analysis of the media showed that the mutant M proteins inhibited VLP assembly in a concentration-dependent manner and that the extent of inhibition increased with an increasing extent of deletion. This is best illustrated by mutant Δ18. At the higher concentration, this protein caused an almost complete block of VLP formation. When its synthesis in cells was reduced to levels that were hardly detectable, VLP production became evident again, but the efficiency was severely decreased. Quantitation showed that VLP assembly increased 12 times when the concentration of Δ18 was about 3 times lower (Fig. 8). No mutant Δ18 protein could be detected in the medium. Similar observations were made with the mutants Δ5 and Δ2, but the effects became progressively weaker as the deletion was made smaller. Finally, the lack of just 1 terminal residue, as in mutant Δ1, did not show any measurable inhibitory effects on VLP formation.

Rescue of deletion mutants into VLPs. Our inability to discriminate between the WT M protein and the mutant M proteins in these competition experiments did not allow an accurate analysis of the possible rescue of the mutant proteins into VLPs. To circumvent this problem and to increase the sensitivity of detection, we made use of mutant A_2A_3, which we showed above to function efficiently in VLP assembly (Fig. 5). Moreover, we found that the mutations in this M protein destroyed the epitope recognized by the monoclonal antibody J1.3 (17). Replacing WT M by this mutant therefore allowed the desired discrimination. Rescue experiments were performed as described above. To allow sufficient VLP production, a 5-times-lower amount of plasmid DNA specifying the M proteins to be rescued was used compared to the amount of A_2A_3 plasmid. Analysis of the cell lysates (Fig. 9) showed that A_2A_3 protein is precipitated with the polyclonal anti-MHV serum k134 but not with the monoclonal antibody J1.3. When mutants Δ18, Δ5, Δ2, or Δ1 or WT M was coexpressed with mutant A_2A_3, M proteins were immunoprecipitated with the polyclonal anti-MHV serum. Only after prolonged exposure could M protein be detected in the samples immunoprecipitated with J1.3, indicating that under the conditions used, the bulk of the expressed M protein is A_2A_3.

Analysis of the media using the polyclonal anti-MHV serum showed that all combinations were productive in VLP formation. Mutant A_2A_3 was easily detected in the medium with the polyclonal serum but not with J1.3 antibodies. Immunoprecipitations with J1.3 showed that mutant M proteins, which were themselves deficient in VLP formation, could be rescued into particles by assembly-competent M. Under the experimental conditions used, A_2A_3 protein in the media was clearly coim-

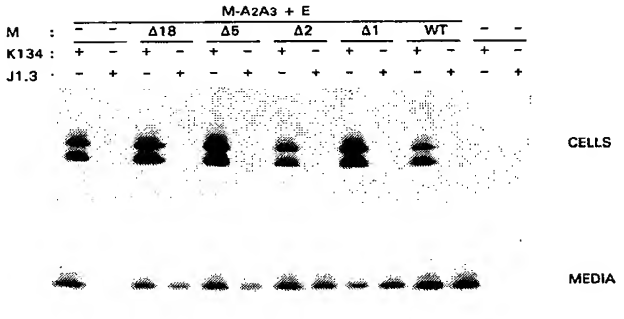

FIG. 9. Rescue of carboxy-terminal deletion mutant M proteins into VLPs. After infection of OST7-1 cells with recombinant vaccinia virus vTF7-3, cells were transfected with plasmid DNA encoding mutant M protein A_2A_3 and E protein (5 μg of each). In some cases, these were cotransfected with 1 μg of plasmid DNA encoding the indicated mutant or WT M protein. Cells were labeled for 3 h with ^{35}S-labeled amino acids. Both cells (upper panel) and the culture medium (lower panel) were prepared and used for immunoprecipitation, either with the polyclonal anti-MHV serum k134 or with the monoclonal antibody J1.3, and the precipitates were analyzed by SDS-PAGE.

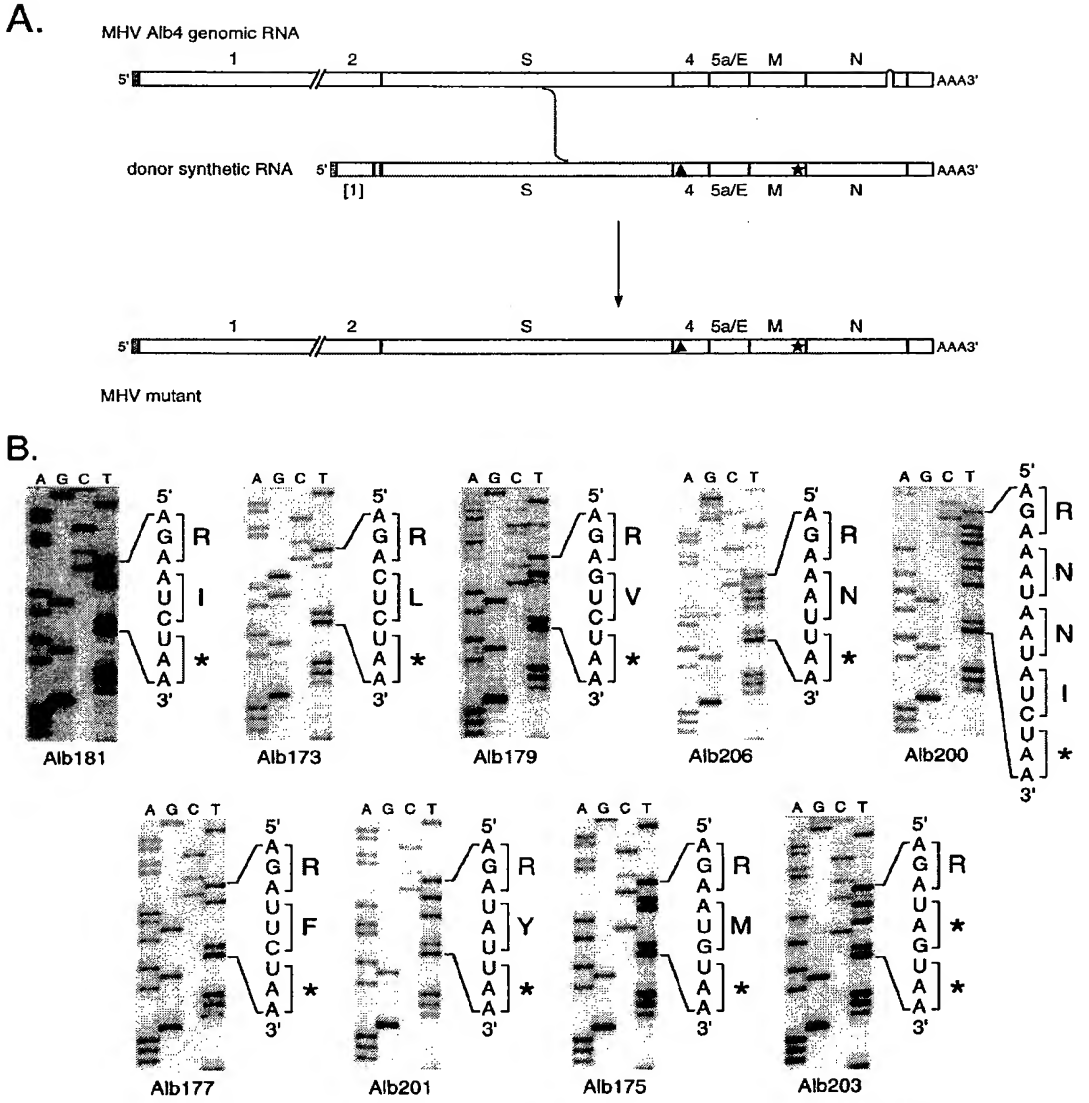

FIG. 10. Incorporation of M protein cytoplasmic-domain mutations into the MHV genome. (A) Schematic for construction of M mutants by targeted recombination between donor synthetic RNA and the N-gene deletion mutant Alb4. Each donor RNA containing a codon 228 mutation in the M gene (denoted by a star) was transcribed from a vector derived from pCFS8 (16), which includes the entire portion of the MHV genome 3′ to the start of the S gene and is tagged with a 19-base marker in gene 4 (denoted by a triangle). In the case shown, the MHV mutant, generated by a single upstream crossover, has inherited the gene 4 tag, the constructed M mutation, and the region repairing the N-gene deletion. (B) Sequence of the relevant region of genomic RNA isolated from passage-3 purified virions of one mutant of each type. For each codon 228 mutation, recombinants were obtained that contained and that lacked the upstream gene 4 tag. The particular mutants shown are all positive for the gene 4 tag, except for Alb206 and Alb200. For Alb200, a BCV-MHV chimeric M protein mutant, the donor RNA was derived from pFV1 (16) and did not contain the gene 4 tag.

munoprecipitated with the deletion mutant proteins, although these proteins themselves were synthesized at relatively low levels. Coimmunoprecipitation of A_2A_3 was interpreted as a measure for rescue of assembly-incompetent M proteins. It was more pronounced when the truncation was smaller, but even mutant Δ18 could still be rescued. These observations provide evidence for the existence of intermolecular interactions between M proteins in the viral particles.

Incorporation of cytoplasmic domain mutations into virions. To further our understanding of the consequences of M protein carboxy-terminal tail mutations in the presence of the full complement of virion structural components, we sought to directly introduce many of these mutations into the genome of MHV A59. This was accomplished by targeted RNA recombination between a synthetic defective interfering (DI) RNA analog containing one of the intended mutations and the N-gene deletion mutant Alb4 as the recipient virus (16, 45, 59) (Fig. 10A). In this manner we were able to isolate several recombinant viruses containing each of the carboxy-terminal residue substitutions that had been studied in the VLP system: $T_{228}I$, $T_{228}L$, $T_{228}V$, and $T_{228}N$ (Fig. 10B). Another recombinant was constructed in which the carboxy-terminal half of the

MHV M protein (amino acid residues 134 to 228) was replaced with its counterpart from BCV (residues 133 to 230), a region containing 17 amino acid differences from MHV, including the residues $NNI_{228-230}$ in place of T_{228} (Fig. 10B). In addition, the substitution mutants $T_{228}F$, $T_{228}Y$, and $T_{228}M$ were also created in the viral M protein (Fig. 10B). (The $T_{228}F$ mutant also contained a secondary mutation, $T_{228}I$.) With one exception, all of these M protein mutants had plaque sizes indistinguishable from that of the WT virus, and at passage 3 all had high yields in tissue culture ($>10^8$ PFU/ml), comparable to or better than that of the WT. For a subset of the mutants, $T_{228}I$, $T_{228}V$, and $T_{228}N$, virus stocks were serially propagated for six passages. Following every passage the genetic stability of each mutant was monitored by direct sequencing of an RT-PCR product of the region containing the constructed mutation. None of the sequences showed any detectable reversion or other secondary genetic alteration, indicating that the introduced genomic changes were stable for at least six passages. The only phenotypic difference noted was with $T_{228}V$ mutants, which, for both of the independent recombinants analyzed, exhibited a slightly smaller plaque size than the WT at 39°C. These results showed that assembly of the complete virion of MHV tolerates changes in the carboxy-terminal residue of the M protein that are considerably more deleterious for the formation of particles from just the M and E proteins (Fig. 4). In the most extreme case, the $T_{228}N$ mutation, which severely diminished VLP production, had no obvious phenotypic effect when incorporated into MHV. As can be seen in Fig. 10B, direct sequencing of RNA isolated from highly purified virions of the $T_{228}N$ mutant Alb206 revealed a substantial amount of overlapping sequence from the leader region of subgenomic RNA 7, which was read by the same primer. This was observed in multiple independent preparations of purified virions of this mutant and of an independent $T_{228}N$ mutant, Alb205. For Alb205, we confirmed the presence of the mutant asparagine codon by sequencing an RT-PCR product from the M-N junction region of the viral genome. We have previously noted that highly purified MHV contains a small amount of packaged subgenomic RNAs (60). It remains for further work to determine if the $T_{228}N$ mutants aberrantly package greater amounts of these RNA species.

We next attempted to introduce the Δ1, Δ2, Δ5, and Δ18 mutations into MHV to investigate their effects on the stability and assembly of virions. These mutants were designed by replacing the respective residues from the carboxy terminus of M with one stop codon (for Δ1 and Δ2) or two stop codons in tandem (for Δ5 and Δ18). This approach was taken in order to avoid unintended effects on the transcription of the downstream N gene that might have resulted from the actual deletion of RNA sequences at the 3′ terminus of the M gene adjacent to the M-N intergenic sequence. Somewhat surprisingly, we were able to isolate recombinants containing the Δ1 mutation (Fig. 10B), which had been lethal for VLP assembly (Fig. 2). This mutant was not only viable but was phenotypically indistinguishable from the WT in plaque size and in viral yield, and it exhibited no detectable accumulation of revertants after six passages. This again points to the existence of interactions in the complete assembled virion that must promote further stability beyond that obtained in VLPs composed solely of M and E proteins.

By contrast, in multiple targeted recombination experiments, we were not able to construct recombinants harboring either the Δ2, Δ5, or Δ18 mutation. In each case, recombinant candidates obtained with these donor synthetic RNAs were analyzed for the acquisition of three markers: (i) repair of the Alb4 N-gene deletion, (ii) the M-gene mutation, and (iii) a phenotypically silent 19-base tag in gene 4 (16) (Fig. 10A). Of 24 recombinants arising from four independent experiments with the Δ2 mutant donor RNA, all of which had repaired the N-gene deletion, none contained either the Δ2 mutation or the gene 4 tag. This suggests, but does not prove, that the Δ2 mutation is lethal to the virus. It is currently unclear why we have not yet obtained gene 4-tagged recombinants from the Δ2 mutant donor RNA. Experiments now underway are aimed at the possibility of recovering the Δ2 mutant if it has a conditional lethal phenotype. Of 12 recombinant viruses obtained from four independent experiments with the Δ5 mutant donor RNA, all had repaired the N-gene deletion but none contained the Δ5 mutation. Most notably, 7 of the 12 also had the gene 4 tag, indicating that each of these must have been derived from at least three crossover events between the donor RNA and the recipient Alb4 genome (Fig. 10A). Similarly, 12 recombinants representing four independent sets from the Δ18 mutant donor RNA did not contain the Δ18 mutation, although all had repaired the N-gene deletion, and 5 of the 12 also had the gene 4 tag. This presence of inherited markers both upstream and downstream of the excluded M-gene mutations provides compelling evidence that the Δ5 and Δ18 mutations are lethal to the virus. Thus, in agreement with the results generated in the VLP system, the carboxy terminus of the M protein must also play a critical role in assembly of the whole virion.

DISCUSSION

Despite a high degree of sequence variation among the coronavirus M proteins, there is a surprising conservation of their overall chemical features (reviewed by Rottier [62]). The dominant common feature is the occurrence of three hydrophobic domains alternating with short hydrophilic regions. The carboxy-terminal half of all M proteins is largely amphiphilic, with a hydrophilic end. This conservation indicates that there are rigid structural constraints on M as a result of functional requirements. Indeed, M has been shown to play a key role in coronavirus assembly. M and E are the only requirements for particle formation (73). Using an envelope assembly assay, we have now shown that all domains of the M protein are important. Changes in the primary structure of the luminal domain, the transmembrane cluster, the amphiphilic domain, and the carboxy-terminal domain had effects on the assembly of M into enveloped particles. Clearly, in order for M to function in assembly, stringent requirements must be satisfied.

The carboxy-terminal domain is located in the cytoplasm. In infected cells this domain is probably important for virus assembly by interacting with the nucleocapsid. An affinity of M for nucleocapsids has been observed in vitro with several coronaviruses. Subviral particles prepared by NP-40 disruption of purified MHV (70, 75), avian IBV (39), or BCV (32) still contained M protein associated with the nucleocapsids. Our results indicate that this cytoplasmic domain is also of crucial importance for the assembly of the viral envelope. Both the exposed carboxy end and the amphiphilic, protease-resistant domain between this end and the transmembrane domains appeared to be essential structural requirements. Strikingly, the extreme carboxy-terminal residues were found to be crucial. The VLP system provided the most sensitive indicator of this: deletion of as little as the single terminal residue of M protein (Δ1) was fatal, while substitutions or extensions at this position generally had a strong negative effect on assembly. Relative to this, in the whole virus the threshold for loss of function was slightly displaced. The Δ1 mutation had no detectable effect when incorporated into the MHV genome, and

only one of eight different substitutions of the carboxy-terminal residue had a weakly measurable impact on viral phenotype. These results suggest that in the complete virion an additional component, most likely the nucleocapsid, provides a further measure of stabilization not available in VLPs composed purely of M and E proteins. Moreover, there may be other factors, such as the kinetics of virion assembly or the relative or absolute levels of viral protein expression, that render the whole virus more tolerant to mutational changes that are devastating in the VLP system. Nevertheless, further truncation of the M protein carboxy terminus in the virion, as in the VLP system, appeared to abrogate its function. In multiple attempts, we were unable to incorporate the Δ2, Δ5, or Δ18 mutation into the MHV genome, suggesting that these mutations, if not absolutely lethal, were at least as harmful to the virus as the lesion in Alb4, the N deletion mutant from which recombinants were selected. These observations all point to a sensitive role of the extreme terminal residues in some aspect of envelope assembly. The exact nature of this role remains unclear, but it is not unlikely that the very carboxy terminus is involved in interactions either intramolecularly, to establish a particular secondary structure in the M molecule, or intermolecularly, with membrane lipids, with E, or with other M proteins. A role of the carboxy terminus in homotypic interactions is, however, unlikely in view of earlier data. Using sucrose gradient analysis, we have shown previously that the MHV M protein alone can associate into large homomeric complexes (36), but this oligomerization still occurred when the carboxy-terminal 22 residues of the protein were deleted.

The cytoplasmic domain of envelope proteins has been assigned an important role in the assembly of many enveloped viruses. It was shown to be essential for incorporation of the glycoprotein of vesicular stomatitis virus (57, 76). For alphaviruses the cytoplasmic domain of the E2 envelope glycoprotein appeared to be critical, with particular roles for a tyrosine, a leucine, and a set of cysteines important for palmitoylation (29, 56, 78). Interestingly, the 2-residue cytoplasmic tail of glycoprotein E1 is dispensable for virus growth (4). In the case of retroviruses, a direct interaction in vitro was reported between the matrix protein and the cytoplasmic domain of the Env protein of human immunodeficiency virus type 1 (HIV-1) (8). Deletions in the cytoplasmic domain of the Env protein of HIV-1 (11, 18, 77) and murine leukemia virus (24) affect Env incorporation into virions. Remarkably, HIV-1 Env mutants with large cytoplasmic deletions can be incorporated into virions in a matrix-independent manner (19, 43). The influenza virus hemagglutinin (HA) cytoplasmic tail is not essential for virus assembly (30, 52, 68). However, deletion of the cytoplasmic tail of influenza virus neuraminidase (NA) severely compromised the incorporation of mutant NA molecules into virions (5, 49).

The amino-terminal domain of the coronaviral M protein is exposed luminally in cellular organelles. This domain varies considerably in length, from some 16 residues in the mature protein of the human coronavirus (HCV) 229E to around 36 in that of feline coronaviruses (see reference 62). No function has been assigned to it yet. Somewhat to our surprise, some changes in this domain did affect MHV VLP formation. At the very amino terminus, insertion of a single residue (alanine) following the initiating methionine was allowed, but a six-His stretch at this position was nearly fatal. Changes in the clusters of hydroxyl amino acids flanking the methionine, or substitutions of the two nearby prolines, were without effect. In contrast, deletion of the middle part of the ectodomain or some substitutions in this domain had severe effects. One such substitution involved a fortuitously obtained tryptophan-to-cysteine mutation at a position close to that where a cysteine naturally occurs in the HCV 229E M protein. While the latter cysteine gives rise to the formation of homodimers (3), no disulfide bonds were observed with the MHV M mutant protein. For alphaviruses, mutations in the ectodomain of the envelope proteins have been shown to affect virus assembly (12, 25, 41). It was suggested that these mutations resulted in impaired lateral interactions causing the budding defect. This might also be the explanation for the behavior of the amino-terminal M mutants.

Coronavirus M proteins are invariably glycosylated, carrying either N-linked or O-linked oligosaccharides (see reference 62). The oligosaccharides are attached to the amino-terminal region of the M molecule and are thus exposed at the virion surface. Since a function for this modification has not been identified, we used our VLP system to study the possible involvement of glycosylation in virus assembly. Our results showed that for MHV, O glycosylation of the M protein is not required. We then created an N-glycosylated form of the protein which appeared still to produce particles, though with decreased efficiency. Only when the maturation of the N-linked oligosaccharide was inhibited did VLP production become normal, indicating that N glycosylation per se was not interfering with assembly. Altogether, these observations demonstrate that glycosylation plays no role in assembly, consistent with the findings of earlier studies that used tunicamycin (69) and monensin (53) to inhibit glycosylation in infected cells. In addition, Laude et al. (40) isolated a mutant of the porcine TGEV in which the sole N-glycosylation site of the M protein had been disrupted without affecting the viability of the virus.

As mentioned above, homotypic interactions between M molecules are supposed to be essential in coronavirus envelope formation. It was therefore of interest to investigate whether M molecules that are themselves deficient in VLP formation would interfere with the formation of particles by assembly-competent M and E. From the set of carboxy-terminal tail deletion mutants of M that we studied, it was clear that interference indeed occurred, in a concentration-dependent manner. Furthermore, assembly-competent M protein was able to rescue assembly-negative M molecules into particles. These observations support the importance of lateral interactions between M molecules in the assembly process. Moreover, they also support our earlier conclusion that such interactions do not require the cytoplasmic tail of the M protein. This conclusion was based on the finding that a mutant M protein lacking the carboxy-terminal 22 amino acids was able to associate into large heterogeneous complexes as does WT M, as shown by sucrose density gradient analysis (36). Lateral interactions between envelope proteins have similarly been demonstrated for Semliki Forest virus. Here it was shown that nucleocapsid binding-deficient p62-E1 heterodimers did inhibit normal virus budding in a concentration-dependent way and that under suitable conditions these heterodimers could be rescued into virus particles (13). It is quite likely that lateral interactions between viral glycoproteins generally are instrumental in the assembly of enveloped viruses.

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Exhibit F

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Role of Plasma Membrane Lipid Microdomains in Respiratory Syncytial Virus Filament Formation

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The fusion protein (F) of respiratory syncytial virus (RSV) is the envelope glycoprotein responsible for the characteristic cytopathology of syncytium formation. RSV has been shown to bud from selective areas of the plasma membrane as pleomorphic virions, including both filamentous and round particles. With immunofluorescent microscopy, we demonstrated evidence of RSV filaments incorporating the fusion protein F and colocalizing with a lipid microdomain-specific fluorescent dye, 1,1-dihexadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate. Western blot analysis of Triton X-100 cold-extracted membrane fractions confirmed the presence of RSV proteins within the lipid microdomains. RSV proteins also colocalized with cellular proteins associated with lipid microdomains, caveolin-1, and CD44, as well as with RhoA, a small GTPase. ADP-ribosylation of RhoA by *Clostridium botulinum* exotoxin inactivated RhoA signaling and resulted in the absence of RSV-induced syncytia despite no significant change in viral titer. We demonstrated an overall decrease in both the number and length of the viral filaments and a shift in the localization of F to nonlipid microdomain regions of the membrane in the presence of C3 toxin. This suggests that the selective incorporation of RSV proteins into lipid microdomains during virus assembly may lead to critical interactions of F with cellular proteins, resulting in microvillus projections necessary for the formation of filamentous virus particles and syncytium formation. Thus, manipulation of membrane lipid microdomains may lead to alterations in the production of viral filaments and RSV pathogenesis and provide a new pharmacologic target for RSV therapy.

Human respiratory syncytial virus (RSV) belongs to the *Pneumovirus* genus of the *Paramyxoviridae* family and is the leading cause of viral respiratory illness in children, accounting for over 100,000 hospitalizations in the United States yearly (37). More recently, RSV has been recognized as a significant pathogen among the elderly (15), nursing home patients (8), and bone marrow and lung transplant recipients (16, 30, 42). In bone marrow transplant recipients infected with RSV, the pathology involves extensive syncytium formation, described as giant-cell pneumonia, for which mortality rates approach 80% (16). Given the significant morbidity and mortality, the development of new antiviral agents for both RSV prophylaxis and therapy is an area of clinical importance and active research.

The RSV virion consists of a lipid envelope that incorporates three transmembrane glycoproteins, F, G, and SH. The G protein is thought to be involved in viral attachment (20), possibly via its binding to cellular sulfated heparin-like glycosaminoglycans (10, 19). The fusion protein F is essential for both virus-to-cell fusion and cell-to-cell fusion, demonstrated by inhibition of syncytium formation with monoclonal antibodies against F (41). Recombinant viruses expressing only the RSV F protein and a mutant RSV virus, cp-52, lacking the G and SH genes are capable of forming syncytia, which provides further evidence for the important role of F in virus entry and RSV pathogenesis (17, 40).

The F protein has been shown to interact with RhoA, a small GTPase binding protein in the Ras superfamily (31). RhoA is ubiquitous in mammalian cells and is involved in a variety of cellular functions, including gene transcription, cell cycle progression, cell morphology, and actin reorganization (18, 25). RhoA requires activation that involves the exchange of GDP for GTP, followed by geranylgeranylation at the carboxy terminus before translocation to the inner leaflet of the plasma membrane (1). Inhibitors of RhoA isoprenylation, including 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have been demonstrated to decrease RSV infection in vitro and in vivo (12).

RhoA activation initiates multiple signaling pathways, leading to effects on cell function and structure, including the formation of actin stress fibers and focal adhesions (33). In vitro, RSV infection activates RhoA and increases membrane-bound RhoA, resulting in an increase in stress fiber formation (11). In addition, RSV-induced syncytium formation has been shown to be dependent on RhoA signaling activity, and viral filament formation appears to be required for syncytium formation (32; T. L. Gower, M. K. Pastey, M. Peebles, P. Collins, T. K. Hart, A. Guth, and B. S. Graham, submitted for publication).

The F protein has been demonstrated to interact with RhoA in vitro. RSV-induced syncytium formation is inhibited by a RhoA-derived peptide, and treatment with the RhoA-derived peptide inhibits RSV infection in an in vivo mouse model (32). While this suggests a critical interaction between the F protein and RhoA in RSV pathogenesis, the exact events involved are not known.

Lipid microdomains, or lipid rafts, are highly liquid-ordered areas of the plasma membrane that are rich in cholesterol and sphingolipids and are insoluble in nonionic detergents such as Triton X-100 at low temperatures (38). Recent studies suggest

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that cell sorting and cellular signaling may occur selectively through these areas (4). Lipid microdomains are enriched in glycosylphosphatidylinositol-anchored proteins and acylated proteins, including the transmembrane influenza virus hemagglutinin (HA) protein and caveolin-1. The association of the HA protein with lipid microdomains has led to the understanding of the preferred budding of influenza virus from lipid rafts (36, 45).

Other viruses have subsequently been shown to selectively bud from lipid microdomains, including another member of the *Paramyxoviridae* family, measles virus, Ebola virus, and human immunodeficiency virus (HIV) (3, 24, 26). Through manipulation of the cholesterol content of the plasma membrane, HIV virus production and syncytium formation can be reduced (21, 23, 28). Since RSV F has properties similar to those of the fusion protein of HIV, gp41, we hypothesized that RSV assembly and budding also occur in lipid rafts and that F may potentially interact with membrane-bound RhoA within these specialized areas of the host cell.

In this study, we sought to identify the cellular association of RSV proteins and host proteins within the lipid microdomain. By using sucrose gradient centrifugation and immunofluorescent microscopy, colocalization of the F protein and RhoA was shown to occur within the lipid raft fraction of the plasma membrane. In addition, CD44 transmembrane protein colocalized with F and RhoA. These interactions may be paramount to viral filament formation, which is necessary for RSV-induced syncytium formation.

MATERIALS AND METHODS

Virus and cells. The A2 strain of RSV was originally provided by R. Chanock, National Institutes of Health, Bethesda, Md. RSV stocks were prepared as previously described (13). HEp-2 cells were maintained in Eagle's minimal essential medium supplemented with glutamine, gentamicin, penicillin G, and 10% fetal bovine serum.

Antibodies, dyes, and reagents. Primary antibodies used for immunofluorescence to detect RSV included a rabbit polyclonal antibody to RSV proteins and a mouse monoclonal antibody for the RSV F protein, gifts of James Crowe, Vanderbilt University, Nashville, Tenn. Western blot analysis for RSV proteins was performed with a horseradish peroxidase-conjugated goat polyclonal antibody from Fitzgerald Industries (Concord, Mass.). Primary mouse monoclonal antibody for RSV N protein was purchased from Accurate Chemical (Westbury, N.Y.), CD44 antibody was from Sigma Pharmaceuticals (St. Louis, Mo.), transferrin receptor antibody was from Zymed Labratories (San Francisco, Calif.), and RhoA antibody was from Santa Cruz Biotechnologies (Santa Cruz, Calif.). Caveolin-1 rabbit polyclonal antibody was obtained from BD Transduction (Franklin Lakes, N.J.), and CD44 rabbit polyclonal antibody was from Santa Cruz Biotechnologies. Secondary antibodies included fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin and anti-mouse immunoglobulin antibodies and rhodamine-labeled goat anti-rabbit immunoglobulin and anti-mouse immunoglobulin antibodies (Molecular Probes, Eugene, Oreg.). Dialkylcarbocyanine dyes $DiIC_{12}(3)$ (1,1-didodecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate) and $DiIC_{16}(3)$ (1,1-dihexadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate), used for selective labeling of lipid membranes, were obtained from Molecular Probes (Eugene, Oreg.). *Clostridium botulinum* exotoxin (C3 toxin) was obtained from Calbiochem (La Jolla, Calif.).

Immunofluorescence assay. HEp-2 cells were plated on coverslips in six-well plates and infected with RSV (100 μl of a 4×10^5 PFU/ml stock). Following a 24-h incubation period post-RSV infection, cells were prepared for analysis by immunofluorescent microscopy. Cells were fixed with 3.7% formaldehyde for 10 min, followed by 0.5% Triton X-100 for 10 min, and then incubated with 5% dry milk–phosphate-buffered saline (PBS) for 15 min to reduce nonspecific binding. Fixed cells were then incubated with primary antibodies in 3% dry milk–PBS at a 1:50 to 1:100 dilution for 1 h and washed with PBS three times. Secondary antibodies diluted at 1:200 in 3% dry milk–PBS were added, and cells were incubated for 1 h- followed by washing with PBS three times. After fixing the slides, viewing was performed with Zeiss Axiovert and Axioplan light microscopes at 40× and 100× oil immersion. Fluorescent images were obtained with a Zeiss AttoArc2 lamp and filter sets 41001 and 41002b from Chroma Technology Corporation (Brattleboro, Vt.). Images were obtained and analysis was performed with Adobe Photoshop software; brightness and contrast were adjusted for viewing.

Immunofluorescence with lipid dye analogs. HEp-2 cells were infected with RSV as described above for 24 h. Six-well plates were then placed on ice and washed with cold PBS, and then 500 μl of $DiIC_{12}(3)$ and $DiIC_{16}(3)$ at a 1:100 dilution in ethanol were added to each well for 15 min. Cells were then washed with PBS and fixed with 3.7% formaldehyde as above. Primary and secondary antibodies were then used as indicated.

Detergent extraction and flotation centrifugation. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0 for 1 h at room temperature. Cells were then incubated at 37°C for 24 h and washed with PBS. Prior to sucrose gradient flotation, select monolayers were treated with 10 mM methyl-β-cyclodextrin (Sigma Chemical, St. Louis, Mo.) for 30 min at 37°C. Sucrose gradient flotation was performed as previously described with minor modification (5). Cells were lysed in 2 ml of TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 and protease inhibitors (Roche Diagnostics, Mannheim, Germany) at 4°C for 30 min, followed by centrifugation at 2,000 rpm for 10 min. The postnuclear supernatant was removed, loaded into SW41 tubes, mixed with 2 ml of 80% sucrose, and layered with 5.0 ml of 35% and 3.0 ml of 5% sucrose. The gradient underwent centrifugation at 4°C for 16 h at 35,000 rpm in a Beckman SW41 rotor. Twelve 1-ml fractions were harvested from the top to the bottom of the gradient.

Protein precipitation, analysis, and blotting. Samples were subjected to methanol precipitation according to the protocol of Wessel and Flugge (43). Two-hundred-microliter aliquots were precipitated by adding 0.8 ml of methanol, followed by 0.2 ml of chloroform. After centrifugation, distilled water was then added at 0.6 ml for phase separation, and the sample was vortexed and then centrifuged for 1 min at 13,000 rpm. The upper phase was removed and discarded. Methanol was added at 0.6 ml, and then the protein pellet was obtained by centrifugation at 13,000 rpm for 2 min. Protein was suspended in 5% sodium dodecyl sulfate and quantified with the BCA assay (Pierce, Rockford, Ill.). Equal amounts of protein from aliquots 3 to 11 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and Western blotting. Densitometry was performed with a GS-800 scanner and Quantity One software from Bio-Rad Laboratories (Hercules, Calif.).

Treatment with C3 toxin. HEp-2 cells were grown on coverslips in 24-well plates. Cells were treated with 250 μl of C3 toxin at 30 μg/ml and incubated overnight at 37°C. Medium was removed, and cells were infected with 100 μl of RSV at 4×10^5 PFU/ml. After 1 h at room temperature, 250 μl of C3 toxin-containing medium was replaced, and cells were allowed to incubate at 37°C overnight. At 24 h postinfection, cells were evaluated by immunofluorescent staining as described above.

RESULTS

RSV produces viral filaments that involve the F protein and the lipid microdomain. Previous studies have demonstrated the presence of both filamentous and round particles budding from the surface of RSV-infected cells (9, 34). The filamentous particles resemble microvilli in their diameter (80 to 120 nm) but are longer, varying in length from 4 to 8 μm. Filamentous projections can be seen originating from the cell surface of cells infected with RSV (Fig. 1). The role of viral filaments in RSV infection remains uncertain. However, the use of selective filtration of viral particles suggests that the long filaments may be the more infectious form of RSV (34).

With F-specific antibody, we were able to demonstrate the presence of the F protein on viral filaments by immunofluorescent microscopy. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0 and, 24 h after infection, stained with monoclonal antibody to the F protein (Fig. 2A). The fusion protein was demonstrated on the filamentous projections associated with RSV-infected cells as well as on budding round virions, suggesting a wide distribution of the F protein.

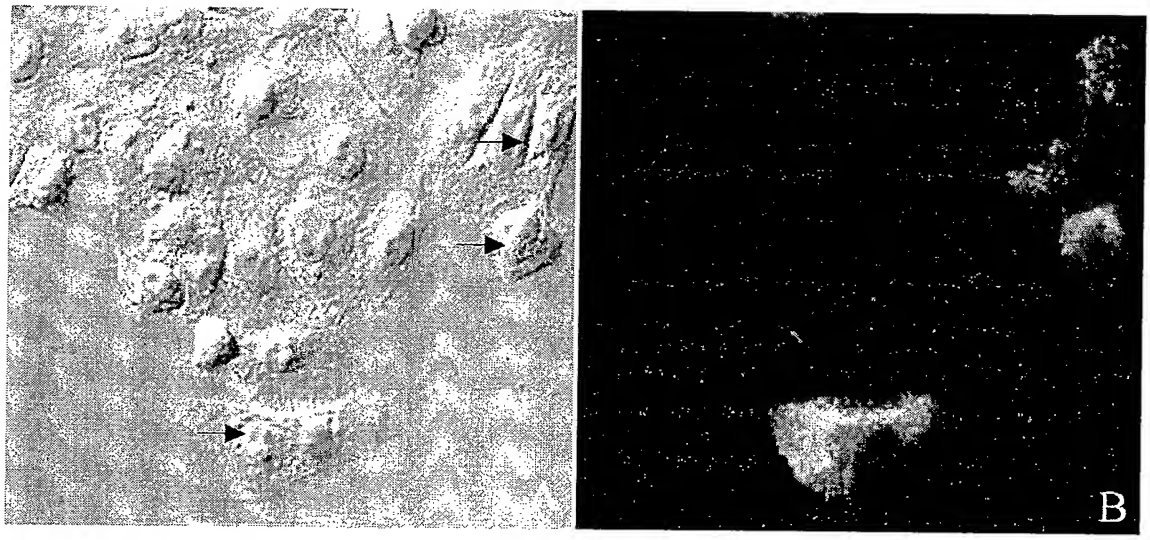


FIG. 1. RSV-infected HEp-2 cells demonstrate morphological changes characterized by filaments which label with anti-RSV antibody. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0 and incubated at 37°C for 24 h. Cells were fixed with 3.7% formaldehyde and blocked with 5% dry milk–PBS. Cells were incubated with rabbit polyclonal anti-RSV antibody followed by FITC-labeled goat anti-rabbit IgG antibody. RSV-infected cells are distinguished by their FITC labeling with fluorescent microcopy (green). The morphology of noninfected and infected (indicated by arrows) was compared by Nomarski differential interference contrast with a 40× objective.

This confirms our findings with electron microscopy, which demonstrate the presence of F on the surface of viral filaments (Gower et al., submitted).

With a monoclonal antibody to the N protein of RSV, we were also able to show the presence of the N protein associated with filamentous structures projecting from the membrane surface (Fig. 2B). The N protein is part of the nucleocapsid, and its identification within the filaments further supports the idea that the filaments represent viral particles budding from the cell surface. $DiIC_{16}(3)$ and $DiIC_{12}(3)$ are long-chain dialkylcarbocyanine fluorescent lipid dyes which partition preferentially into different domains of the plasma membrane. $DiIC_{16}(3)$ has been shown to incorporate in liquid-ordered domains, while $DiIC_{12}(3)$ is incorporated in fluid domains (39). Images of RSV-infected HEp-2 cells stained with $DiIC_{16}(3)$ demonstrated the presence of viral filaments budding from the cell membrane (Fig. 2C), while there was no evidence of viral filaments in $DiIC_{12}(3)$-stained infected cells (not shown).

RSV proteins RhoA and CD44 are isolated from Triton X-100-insoluble membrane in RSV-infected HEp-2 cells. Identification of proteins associated with the lipid microdomain was performed by cell lysis with the detergent Triton X-100 at 4°C, followed by sucrose gradient centrifugation as previously described (5). Selected fractions were then examined for the

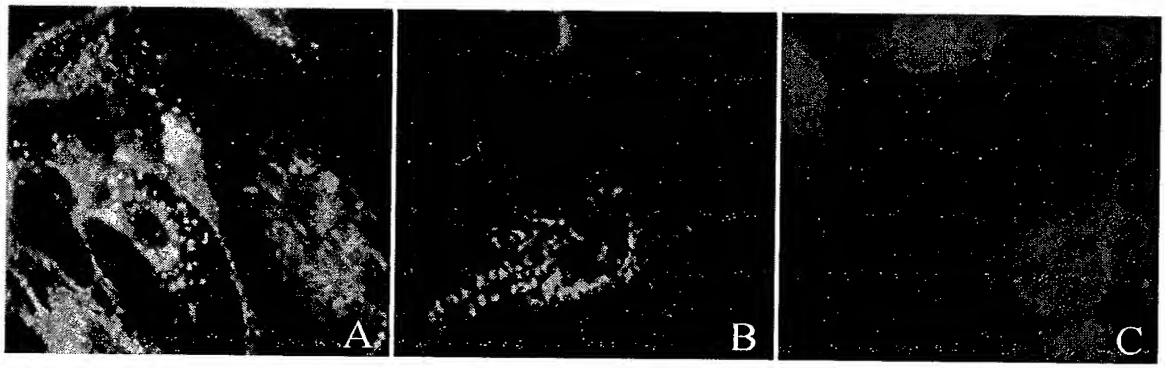


FIG. 2. RSV produces viral filaments which label With both the F and N proteins and with the lipid dye $DiIC_{16}(3)$. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0 and incubated at 37°C for 24 h. Cells were fixed with 3.7% formaldehyde and permeabilized with 0.5% Triton X. After blocking, cells were incubated with mouse anti-F (A) and anti-N (B) monoclonal antibodies followed by FITC-labeled goat anti-mouse IgG (green). In addition, prior to fixation, RSV-infected HEp-2 cells were incubated on ice with either $DiIC_{12}(3)$ (not shown) or $DiIC_{16}(3)$ (C) for 15 min. Cells were then fixed and imaged in the absence of antibody. Filamentous particles observed budding from the surface of RSV-infected cells demonstrate the presence of both the F and N proteins of RSV and incorporated the lipid dye $DiIC_{16}(3)$, which is specific for the lipid raft.

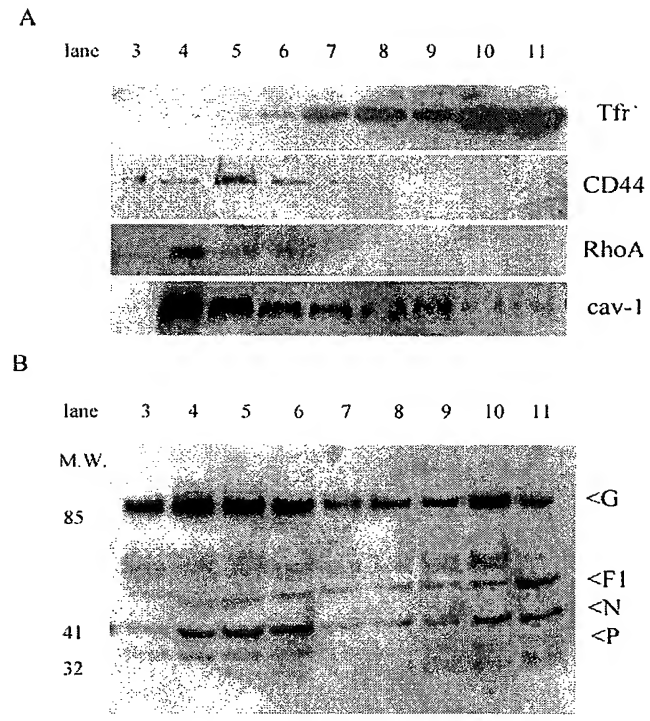


FIG. 3. RSV proteins localize to the detergent-insoluble raft microdomains after sucrose gradient separation. RSV-infected HEp-2 cells were lysed in 1% Triton X-100 at 4°C at 24 h postinfection. Postnuclear extract was layered with a discontinuous sucrose gradient and centrifuged at 35,000 rpm for 16 h, and 1-ml fractions were collected (see Materials and Methods for details). Fraction 1 represents the top of the gradient, 12 represents the bottom, and fractions 3 to 5 are where the cholesterol-rich regions of the membrane localize. Examination of cellular proteins was performed by Western blotting after separation by sodium dodecyl sulfate-PAGE and transfer to polyvinylidene difluoride membranes. (A) CD44 and RhoA localized to fractions 3 to 5 with raft protein caveolin-1 (cav-1), while the nonraft protein transferrin receptor (Tfr) was noted in soluble fractions 8 to 11. (B) Membrane localization of RSV proteins was examined with a goat polyclonal RSV antibody. RSV proteins, including G and F1, are found in both the lipid raft and nonraft fractions, while there is a predominant localization of N and P proteins to the lipid raft domains.

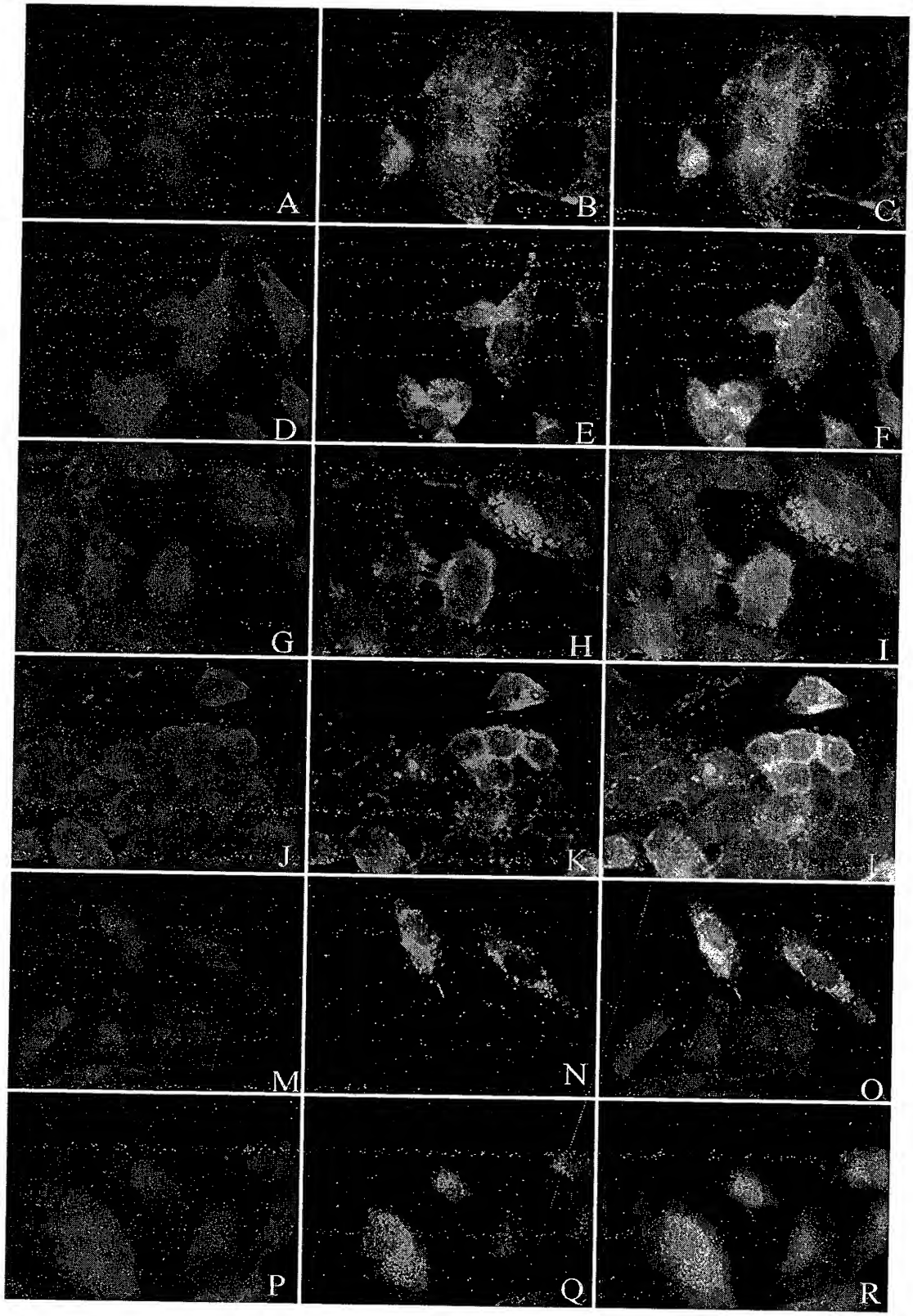
presence of viral and host cellular proteins by Western blot analysis. Immunoblotting of RSV-infected HEp-2 cells demonstrated the presence of both caveolin-1 and CD44 within the Triton X-insoluble fractions (fractions 3 to 5), which corresponds to the raft component of the plasma membrane (Fig. 3A), while the nonraft transferrin receptor was found predominantly within the soluble fractions, 8 to 11 (Fig. 3A). This distribution of caveolin-1 and CD44 within the lipid raft is consistent with previous observations (27).

We then examined the membrane distribution of the GTPase RhoA. Gower et al. have shown that membrane-associated RhoA is increased following RSV infection (11). We showed that 24 h after RSV infection, RhoA migrated with CD44 and was found in detergent-insoluble raft fractions (Fig. 3A). RSV is highly cell associated, and polyclonal antibody directed against RSV demonstrated the presence of viral proteins in both detergent-insoluble and detergent-soluble fractions of the gradient (Fig. 3B). Densitometry performed on Western blots of RSV proteins revealed that a significant fraction of the envelope proteins, F protein (29.9%) and G (61.0%), and the N and P proteins (64.4% and 66.4%, respectively) resided within the lipid microdomain fractions (see Fig. 8B).

Caveolin-1, RhoA, CD44, and F protein colocalize with lipid dye specific for the lipid microdomain. With immunofluorescent markers that selectively partition into liquid-ordered and fluid domains of the plasma membrane, we sought to determine colocalization of viral proteins and cellular proteins with the lipid microdomains. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0 and, 24 h postinfection, were labeled with either the $DiIC_{16}(3)$ or $DiIC_{12}(3)$ dye for 15 min on ice. After fixation, the cells were labeled with primary antibody, followed by FITC-labeled anti-mouse or anti-rabbit IgG. As predicted by Western blot analysis, caveolin-1, CD44, and RhoA (green) demonstrated principal localization to the lipid microdomain by overlapping with the $DiIC_{16}(3)$ (red), shown by the yellow color (Fig. 4F, L, and R, respectively). In contrast, there was minimal overlap between either caveolin-1, CD44, or RhoA with the nonraft dye $DiIC_{12}(3)$, further supporting the localization of these proteins to the lipid microdomains (Fig. 4C, I, and O, respectively).

Cells were also labeled with a monoclonal antibody against the F protein, followed by FITC-labeled anti-mouse IgG. As previously noted, evidence of filamentous projections containing the F protein was again present (Fig. 5B and E). In addition, RSV-infected cells demonstrated aggregated foci of the F protein, suggesting a selective targeting of virus to the plasma membrane, which has been described previously by Bachi with video microscopy (2). Both the filaments and the aggregated areas of F overlapped the raft dye $DiIC_{16}(3)$, as shown in Fig. 5F. The majority of the labeled F colocalized with the lipid microdomain, while there were some areas in which the fusion protein was excluded from the lipid raft, consistent with Western blot analysis. Although the presence of F protein in the nonraft domain is likely based on Western blot analysis, sections of cells stained with $DiIC_{12}(3)$ demonstrated little evidence of overlap with labeled antibody to the F protein (Fig. 5C).

FIG. 4. Colocalization of caveolin-1, CD44, and RhoA with lipid microdomains in immunofluorescent microscopy. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0. At 24 h postinfection, cells were incubated with dialkylindocarbocyanine lipid dye $DiIC_{12}(3)$ (panels A, G, and M) or $DiIC_{16}(3)$ (panels D, J, and P) for 15 min on ice (shown in red). Cells were then fixed and labeled with primary antibody, as indicated, followed by FITC-labeled goat anti-mouse IgG (green) Panels B and E represent anti-caveolin-1, H and K represent anti-CD44, and N and Q represent anti-RhoA. Colocalization of the cellular proteins with the respective lipid dye markers is demonstrated in yellow (panels C, F, I, L, O, and R). Consistent with the Western blot analysis, CD44 and RhoA localized to the lipid raft domain along with caveolin-1 in RSV-infected cells.



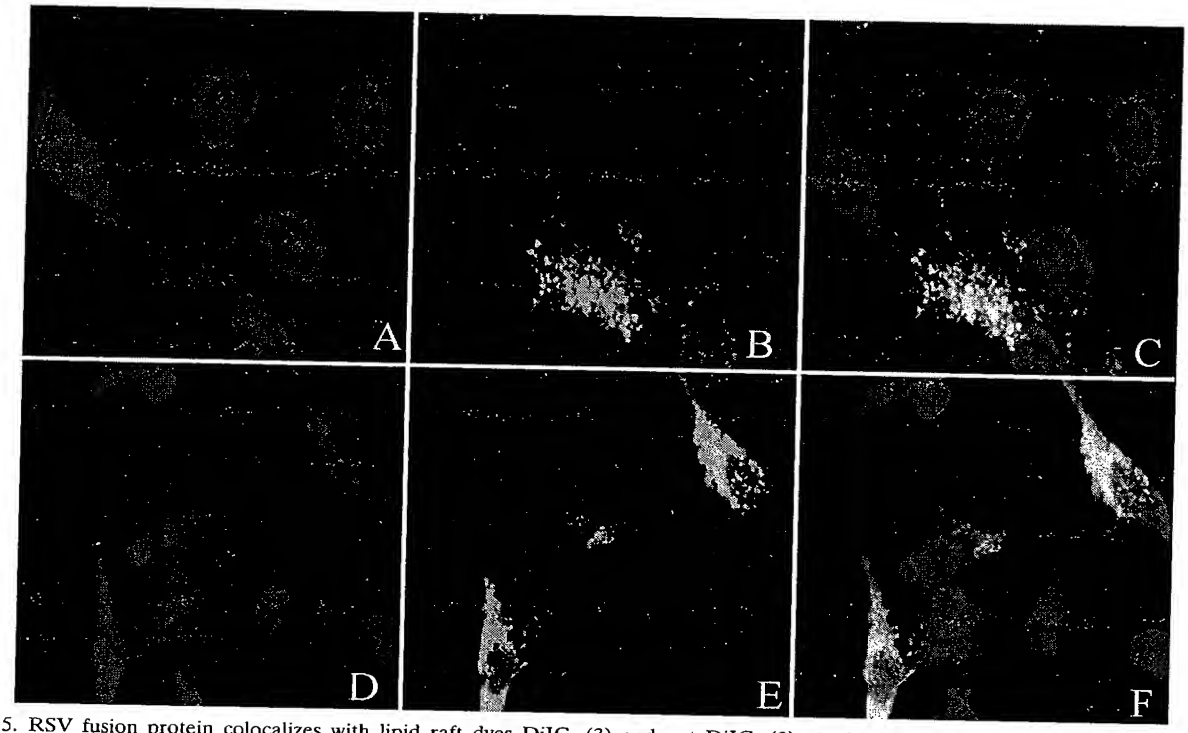


FIG. 5. RSV fusion protein colocalizes with lipid raft dyes $DiIC_{16}(3)$ and not $DiIC_{12}(3)$. At 24 h postinfection, RSV-infected cells were incubated with lipid dye $DiIC_{12}(3)$ (A) or $DiIC_{16}(3)$ (D) for 15 min on ice. Cells were then fixed and labeled with mouse anti-F monoclonal antibody, followed by FITC-labeled anti-mouse IgG, shown in green (B and E). Images demonstrate membrane aggregation of F protein and filament formation. F protein colocalizes preferentially with the lipid dye $DiIC_{16}$ (F) compared to $DiIC_{12}$ (C), as demonstrated in yellow.

RSV proteins overlap caveolin-1, RhoA, and CD44 in infected HEp-2 cells. We used immunofluorescent microscopy to demonstrate colocalization of RSV proteins with RhoA and CD44 (Fig. 6A and D, respectively). RSV proteins labeled with a rabbit polyclonal antibody followed by a secondary FITC-labeled goat anti-rabbit IgG antibody (Fig. 6B) overlapped rhodamine-labeled RhoA (Fig. 6C). With the mouse monoclonal anti-F antibody followed by goat FITC-labeled anti-mouse IgG (Fig. 6E), we also showed overlap with CD44 labeled with a rabbit polyclonal antibody, followed by a rhodamine-labeled goat anti-rabbit IgG (Fig. 6F). This colocalization by immunofluorescent microscopy further supports an interaction of RSV with structural cellular proteins in the lipid microdomains, which was previously demonstrated with caveolin-1 and GM1 (6, 7).

Treatment with C3 toxin alters the morphology of RSV filaments. *Clostridium botulinum* exotoxin (C3 toxin) inhibits effector functions of RhoA. C3 toxin has been shown to block stress fiber formation in RSV-infected HEp-2 cells and to inhibit syncytium formation (11). HEp-2 cells were treated with C3 toxin (30 μg/ml) or left untreated 24 h prior to RSV infection. Cells were then infected with RSV for 1 h and re-treated with C3 toxin or left untreated. Visualization of the cells 24 h postinfection demonstrated a change in the physical characteristics of RSV filaments (Fig. 7B and E). In the presence of C3 toxin, the RSV filaments were both fewer in number and shorter in length. This suggests that while RSV is able to replicate and assemble infectious virions in the absence of RhoA signaling, the assembly and budding of virus are altered.

We next determined the localization of F in C3 toxin-treated cells with the lipid dye analogs $DiIC_{12}(3)$ and $DiIC_{16}(3)$ (Fig. 7A and D). The colocalization of the F protein with the non-lipid dye $DiIC_{12}(3)$ was increased relative to $DiIC_{16}(3)$ in C3-treated RSV-infected cells (Fig. 7C and F) compared to untreated cells (Fig. 5C and F). This suggests the RSV F glycoprotein is not targeted to cholesterol-rich lipid microdomains when RhoA activation is inhibited.

Disruption of lipid microdomains alters the distribution of RSV proteins. Lipid microdomains are characterized by their high content of cholesterol and sphingolipids. Manipulation of the plasma membranes with cyclic oligosaccharides, cyclodextrins, removes cholesterol and disrupts the formation of lipid rafts. After a 24-h infection, we subjected RSV-infected monolayers of HEp-2 cells to 10 mM methyl-β-cyclodextrin for 30 min. Monolayers were then subjected to Triton X-100 at 4°C and layered over a sucrose gradient. Western blot analysis of gradient fractions revealed a nearly complete loss of RSV proteins from the lipid microdomains (Fig. 8A). Densitometry demonstrated a substantial difference in the distribution of RSV proteins compared to that in RSV-infected cells in the absence of cyclodextrin (Fig. 8B). This shows that cholesterol is important to the distribution of viral proteins within the membrane and suggests that manipulation of lipid microdomains may alter the production of infectious virions.

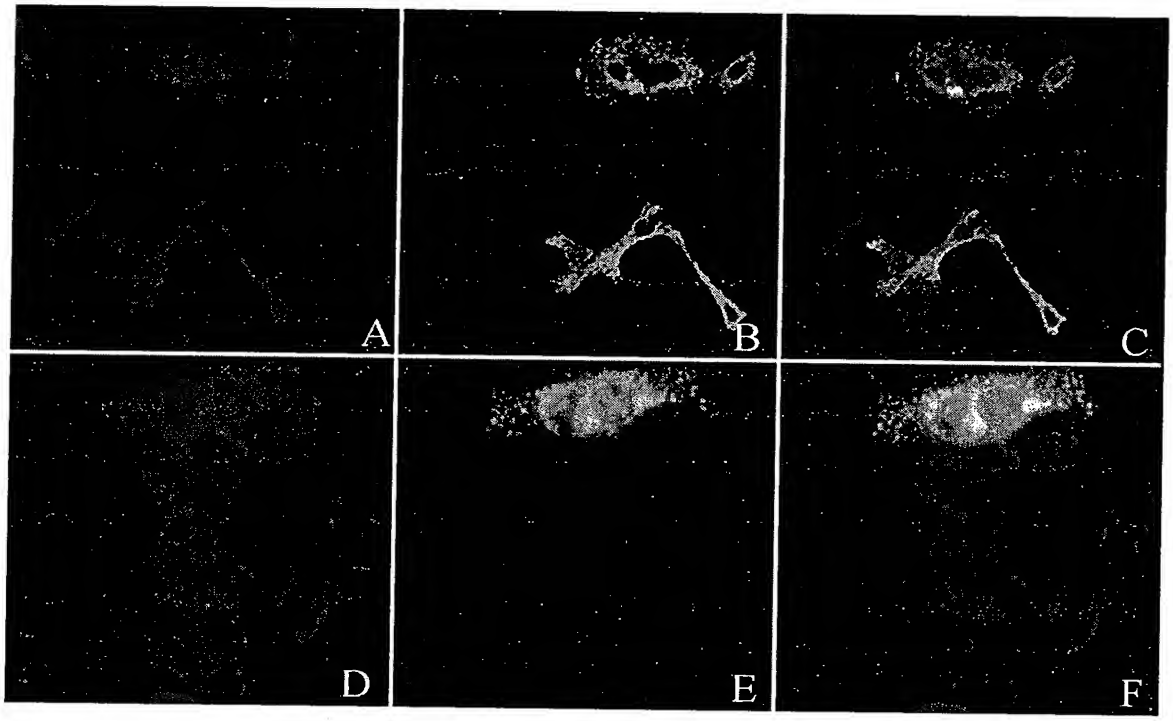


FIG. 6. RSV proteins colocalize with RhoA and CD44. HEp-2 cells were infected with RSV at a multiplicity of infection of 1.0, and 24 h postinfection they were fixed and stained with the primary antibody, mouse monoclonal anti-RhoA (A) or rabbit polyclonal anti-CD44 (D). Simultaneously, cells were stained with either rabbit polyclonal anti-RSV (B) or mouse monoclonal anti-F (E) antibody. Cells were then stained with rhodamine-labeled goat anti-mouse or anti-rabbit IgG (red) and FITC-labeled goat anti-mouse or anti-rabbit IgG (green). The overlap between anti-RhoA and anti-RSV is shown in panel C (yellow) and the overlap between anti-CD44 and anti-RSV is shown in panel F (yellow). Antibodies against both CD44 and RhoA demonstrate overlap with RSV proteins, suggesting colocalization of the cellular proteins with RSV in infected cells.

DISCUSSION

RSV accounts for significant morbidity among infants and selected immunocompromised hosts. Similar to other paramyxoviruses, it is a lipid-enveloped virus which consists of a fusion protein, F, incorporated into the viral membrane, in addition to its other envelope proteins, G and SH. The F protein of RSV is essential for the characteristic cytopathology of RSV, marked by the formation of syncytia. In vitro evaluation of RSV has revealed the presence of pleomorphic viral particles emanating from infected cells, including both round and long filamentous structures. The latter resemble cellular microvilli. With immunofluorescence, we were able to demonstrate the presence of both filaments and round particles labeled with a monoclonal antibody to the F protein in RSV-infected HEp-2 cells. This suggests that the filaments represent viral particles budding from the cell surface microvilli, which is further supported by the presence of the N protein in the filamentous projections and microvilli.

Roberts et al. (34) demonstrated polarized maturation and budding of RSV from the host membrane, suggesting clustering of the virus prior to its release from the host cell. This selective targeting of virus has been demonstrated for other viruses and their proteins, including influenza virus and its HA protein (36, 45). Specifically, influenza virus, measles virus, Ebola virus, and HIV have been shown to incorporate into the lipid microdomains of the host cell and bud selectively from these discrete areas. In this study, we are able to show, through both traditional sucrose density gradients and immunofluorescent microscopy, the association of RSV F glycoprotein with lipid rafts and their constitutive proteins.

Lipid microdomains, or rafts, are highly liquid-ordered areas of the plasma membrane which exist in equilibrium with adjacent nonraft domains. These microdomains are enriched in cholesterol, sphingomyelin, and both glycosylphosphatidylinositol-anchored and acylated proteins. Because of these unique characteristics, lipid rafts can be distinguished from nonraft domains. Nyguen and Hildreth demonstrated selective incorporation of HIV into lipid rafts with dialkylcarbocyanine dyes (26). $DiIC_{16}(3)$ consists of a long acyl chain, which allows its incorporation into the more rigid, ordered lipid domains, compared to $DiIC_{12}(3)$, which labels the fluid nonraft membrane components (39). To demonstrate the selective incorporation of the dyes into their respective membrane compartments, we used antibodies to both caveolin-1 and CD44. These proteins have previously been isolated from lipid rafts, and similarly, we are also able to demonstrate raft distribution of caveolin-1 and CD44 through sucrose density gradients following detergent extraction. With the DiIC dyes, we showed the

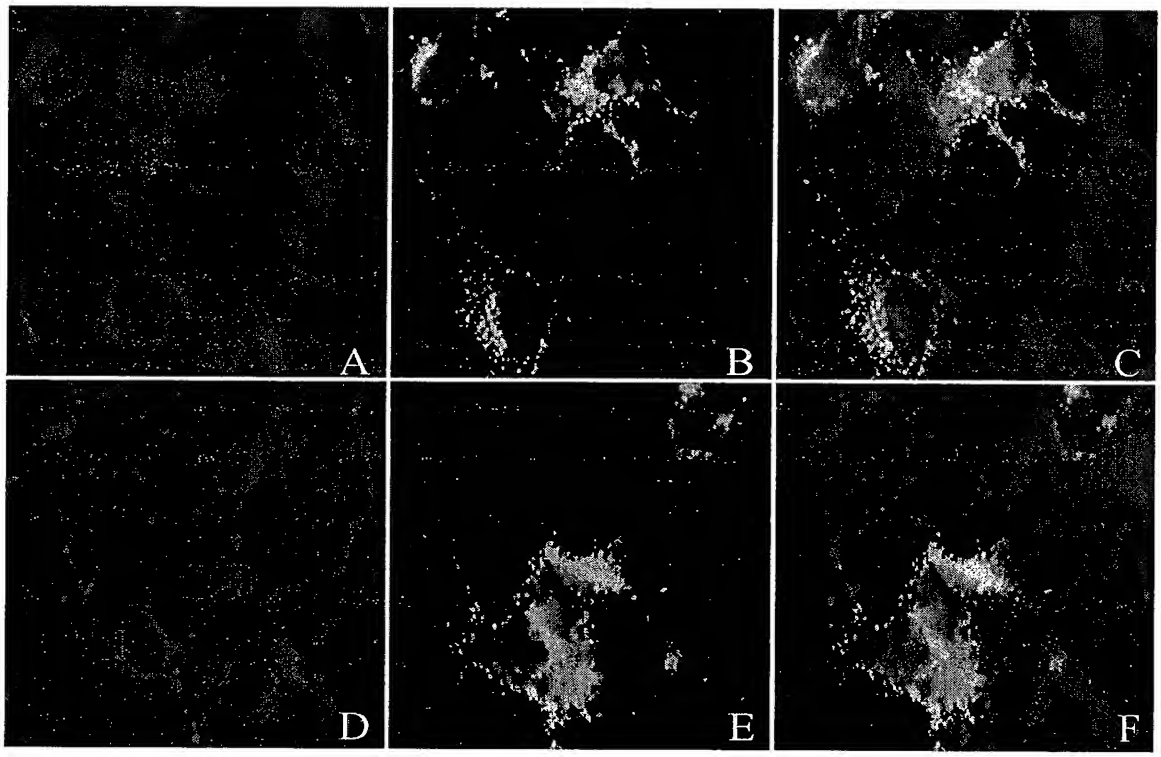


FIG. 7. Treatment of HEp-2 cells with C3 toxin prior to RSV infection blunts filament formation and alters the colocalization of F with the lipid microdomain dye. HEp-2 cells were treated with 250 μl of C3 toxin at 30 μg/ml overnight prior to infection with RSV. Cells were infected with RSV at a multiplicity of infection of 1.0 and then incubated for 24 h after RSV infection in the presence or absence of C3 toxin. Cells were then incubated with lipid dye $DiIC_{12}(3)$ (A) or $DiIC_{16}(3)$ (D) on ice (red). Cells were fixed and labeled with mouse anti-F monoclonal antibody, followed by FITC-labeled mouse anti-IgG antibody (B and E; green). Overlap between the dyes and anti-F is shown in yellow (C and F). Following treatment with C3 toxin, the viral filaments of RSV-infected cells appear shortened and relatively decreased in number. In addition, the overlap between the F protein and $DiIC_{12}(3)$, the nonraft dye, is increased, while there is diminished localization with the lipid microdomain relative to untreated cells.

overlap between the two proteins with $DiIC_{16}(3)$ and their exclusion from areas labeled with $DiIC_{12}(3)$.

Examination of images of $DiIC_{16}(3)$-stained cells demonstrated the labeling of viral filaments, while there is no evidence of filaments in $DiIC_{12}(3)$-stained cells. This suggests that RSV filaments may originate from selected areas of the membrane involving the lipid raft. Brown and colleagues recently demonstrated the presence of a lipid raft protein, caveolin-1, on the surface of budding RSV filaments by both immunofluorescent and electron microscopy (6). With monoclonal antibodies to the F protein, we were able to confirm the aggregation of F protein within the lipid rafts by showing predominant colocalization of F with the lipid microdomain dye, with nearly complete exclusion of F from the nonraft domain. While there were some areas of F staining which did not overlap with the lipid dye, and likewise few areas which overlapped with $DiIC_{12}(3)$, this finding can be explained by the highly membrane-associated nature of RSV.

Up to 90% of RSV remains cell associated, and subsequently one would anticipate overlap of RSV proteins with the nonraft domains, as the raft portion of the host membrane is the minority. This finding was also noted in Western blot analysis of RSV proteins, where viral proteins were associated with both detergent-insoluble and -soluble components of the membrane, including the envelope protein F. However, there was evidence that certain viral proteins are found predominantly in the lipid raft microdomains, including the G protein and the nucleocapsid proteins N and P. Selective targeting of F and the nucleocapsid proteins to the microdomain may initiate events that favor assembly of RSV virions in the lipid rafts. As has been suggested for HIV, coalescence of lipid raft domains at the time of virus budding may approximate viral proteins and lead to a higher density of F glycoprotein expression on infectious viral progeny. Similarly, the selective targeting of F and other RSV proteins to the cell membrane may initiate events that lead to the formation of microvillus projections and subsequent release of filamentous RSV virions from microdomains.

The interaction of actin with other cytoskeleton components is important for the formation of microvillus projections. Previous work has demonstrated a role for CD44 and the ERM (ezrin, radixin, and moesin) family of proteins in microvillus formation via actin cross-linking (22, 44). RhoA is a small GTPase which influences the formation of actin stress fibers and also leads to microvillus formation upon phosphorylation of the ERM proteins (29). In vitro, RhoA has been shown to

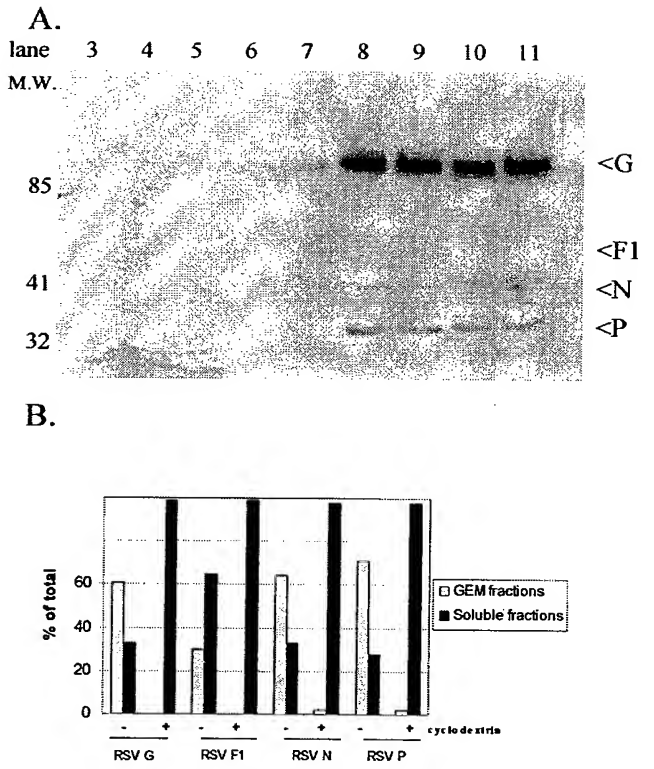


FIG. 8. Treatment of RSV-infected cells with methyl-β-cyclodextrin alters the distribution of RSV proteins within the lipid microdomains. At 24 h postinfection, RSV-infected HEp-2 cells were treated with 10 mM methyl-β-cyclodextrin for 30 min at 37°C and then lysed in 1% Triton X-100 at 4°C. Postnuclear extract was layered with a discontinuous sucrose gradient and centrifuged at 35,000 rpm for 16 h, and 1-ml fractions were collected. Fraction 1 represents the top of the gradient, and fraction 12 represents the bottom. Examination of viral proteins with a horseradish peroxidase-conjugated goat polyclonal anti-RSV antibody was performed by Western blotting after separation by sodium dodecyl sulfate-PAGE and transfer to polyvinylidene difluoride membranes (A). Quantification of viral proteins by densitometry was performed for both untreated (Fig. 3B) and cyclodextrin-treated lysates following sucrose gradient separation, and the percentage of each individual protein distributed to the lipid microdomains (fractions 3 to 6) was determined (B). RSV envelope and nucleoproteins are found within the lipid microdomain fractions. Comparison of untreated RSV-infected HEp-2 cells to cyclodextrin-treated cells demonstrates a substantial alteration in the distribution of RSV proteins following manipulation of the cholesterol content.

interact with the RSV F protein (31). In addition, RSV infection leads to both translocation of activated RhoA to the plasma membrane and a RhoA-dependent increase in stress fiber formation (11). While the in vivo relationship between RhoA and F has not been determined, we showed that RhoA is detergent insoluble at low temperature and partitions to the lipid raft portion of the sucrose gradient by Western blot analysis in RSV-infected cells.

Through immunofluorescent imaging, we confirmed colocalization of RhoA with the lipid raft dye, while showing that it is predominantly excluded from nonraft domains. With polyclonal antibody to RSV, we were also able to demonstrate an overlap of RhoA with the RSV proteins. This suggests that in RSV-infected cells, colocalization of RhoA and F occurs in membrane lipid microdomains. RhoA activation is necessary for filament formation, perhaps through direct effects on CD44 and ERM proteins. Whether a direct interaction of F with RhoA is involved in this process is not known. The possible interaction of the F protein with ERM proteins needs to be investigated further, as the rabies virus and other viruses have been described to incorporate actin-binding proteins into their virions (35).

We further demonstrated the importance of RhoA in RSV filament formation with *C. botulinum* exotoxin. C3 toxin irreversibly inactivated RhoA signaling pathways by ADP-ribosylation of RhoA. RSV-induced stress fiber formation and syncytium formation are inhibited in the presence of C3 treatment, although viral titer is unaffected (11). We showed that the morphological characteristics of RSV filaments are altered in the presence of C3 toxin. Filaments appeared blunted compared to untreated cells. Similarly, electron microscopy of C3 toxin-treated RSV-infected cells showed diminished length and number of budding filaments (Gower et al., submitted for publication). The lack of filaments may be responsible for the absence of syncytia among C3 toxin-treated cells.

In addition, the maturation site of RSV appeared to be changed by C3 toxin, as fluorescently labeled F protein is less associated with the lipid raft dye $DiIC_{16}(3)$ in the presence of C3 toxin compared to control RSV-infected cells and shows a relative increase in colocalization with $DiIC_{12}(3)$. Interference with RhoA activity may lead to abnormal targeting of viral protein to the nonraft domains of the cell surface and a subsequent lack of filaments and syncytia, thereby altering the pathogenesis of RSV. Similarly, we demonstrated that the distribution of viral proteins is significantly altered following the removal of cholesterol from the cellular membrane. Treatment of infected cells with cyclodextrin effectively disassociates RSV proteins from lipid microdomains and provides a possible new mechanism to inhibit the formation of infectious RSV virions.

RSV is an important respiratory pathogen in infants and has increasingly emerged as a cause of severe morbidity and mortality in elderly and immunocompromised hosts. The importance of the RSV F protein to the pathogenesis of syncytium formation has been well characterized, including the cytopathologic development of giant-cell pneumonia in immunocompromised hosts. We demonstrate an association of the F protein with lipid microdomains of the plasma membrane and the presence of viral filaments budding selectively from these specialized regions. As with HIV and other enveloped viruses, it suggests a preferential localization of viral proteins within the lipid microdomains, allowing favorable interactions with cellular proteins, including RhoA and CD44, that then leads to the formation of filaments, responsible for the development of syncytia. Manipulation of either the lipid component of the membrane or the incorporation of cellular or viral proteins into the microdomains may favorably alter RSV pathogenesis and provide future targets for pharmacologic therapy directed at the prevention and treatment of RSV-caused disease.

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Exhibit G

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Antiseptics and Disinfectants: Activity, Action, and Resistance

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INTRODUCTION

Antiseptics and disinfectants are used extensively in hospitals and other health care settings for a variety of topical and hard-surface applications. In particular, they are an essential part of infection control practices and aid in the prevention of nosocomial infections (277, 454). Mounting concerns over the potential for microbial contamination and infection risks in the food and general consumer markets have also led to increased use of antiseptics and disinfectants by the general public. A wide variety of active chemical agents (or "biocides") are found in these products, many of which have been used for hundreds of years for antisepsis, disinfection, and preservation (39). Despite this, less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. The widespread use of antiseptic and disinfectant products has prompted some speculation on the development of microbial resistance, in particular cross-resistance to antibiotics. This review considers what is known about the mode of action of, and mechanisms of microbial resistance to, antiseptics and disinfectants and attempts, wherever possible, to relate current knowledge to the clinical environment.

A summary of the various types of biocides used in antiseptics and disinfectants, their chemical structures, and their clinical uses is shown in Table 1. It is important to note that many of these biocides may be used singly or in combination in a variety of products which vary considerably in activity against microorganisms. Antimicrobial activity can be influenced by many factors such as formulation effects, presence of an organic load, synergy, temperature, dilution, and test method. These issues are beyond the scope of this review and are discussed elsewhere (123, 425, 444, 446, 451).

DEFINITIONS

"Biocide" is a general term describing a chemical agent, usually broad spectrum, that inactivates microorganisms. Because biocides range in antimicrobial activity, other terms may be more specific, including "-static," referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and "-cidal," referring to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal). For the purpose of this review, antibiotics are defined as naturally occurring or synthetic organic substances which inhibit or destroy selective bacteria or other microorganisms, generally at low concentrations; antiseptics are biocides or products that destroy or inhibit the growth of microorganisms in or on living tissue (e.g. health care personnel handwashes and surgical scrubs); and disinfectants are similar but generally are products or biocides that are used on inanimate objects or surfaces. Disinfectants can be sporostatic but are not necessarily sporicidal.

Sterilization refers to a physical or chemical process that completely destroys or removes all microbial life, including spores. Preservation is the prevention of multiplication of microorganisms in formulated products, including pharmaceuticals and foods. A number of biocides are also used for cleaning purposes; cleaning in these cases refers to the physical removal of foreign material from a surface (40).

MECHANISMS OF ACTION

Introduction

Considerable progress has been made in understanding the mechanisms of the antibacterial action of antiseptics and disinfectants (215, 428, 437). By contrast, studies on their modes of action against fungi (426, 436), viruses (298, 307), and protozoa (163) have been rather sparse. Furthermore, little is known about the means whereby these agents inactivate prions (503).

Whatever the type of microbial cell (or entity), it is probable that there is a common sequence of events. This can be envisaged as interaction of the antiseptic or disinfectant with the cell surface followed by penetration into the cell and action at the target site(s). The nature and composition of the surface vary from one cell type (or entity) to another but can also alter as a result of changes in the environment (57, 59). Interaction at the cell surface can produce a significant effect on viability (e.g. with glutaraldehyde) (374, 421), but most antimicrobial agents appear to be active intracellularly (428, 451). The outermost layers of microbial cells can thus have a significant effect on their susceptibility (or insusceptibility) to antiseptics and disinfectants; it is disappointing how little is known about the passage of these antimicrobial agents into different types of microorganisms. Potentiation of activity of most biocides may be achieved by the use of various additives, as shown in later parts of this review.

In this section, the mechanisms of antimicrobial action of a range of chemical agents that are used as antiseptics or disinfectants or both are discussed. Different types of microorganisms are considered, and similarities or differences in the nature of the effect are emphasized. The mechanisms of action are summarized in Table 2.

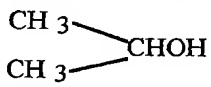
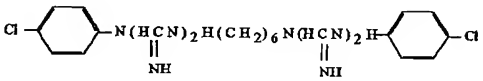
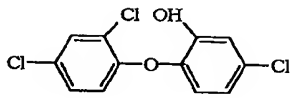
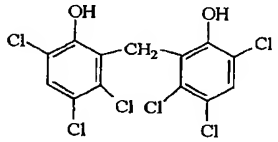
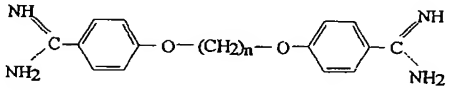
General Methodology

A battery of techniques are available for studying the mechanisms of action of antiseptics and disinfectants on microorganisms, especially bacteria (448). These include examination of uptake (215, 428, 459), lysis and leakage of intracellular constituents (122), perturbation of cell homeostasis (266, 445), effects on model membranes (170), inhibition of enzymes, electron transport, and oxidative phosphorylation (162, 272), interaction with macromolecules (448, 523), effects on macromolecular biosynthetic processes (133), and microscopic examination of biocide-exposed cells (35). Additional and useful information can be obtained by calculating concentration exponents (n values [219, 489]) and relating these to membrane activity (219). Many of these procedures are valuable for detecting and evaluating antiseptics or disinfectants used in combination (146, 147, 202, 210).

Similar techniques have been used to study the activity of antiseptics and disinfectants against fungi, in particular yeasts. Additionally, studies on cell wall porosity (117–119) may provide useful information about intracellular entry of disinfectants and antiseptics (204–208).

Mechanisms of antiprotozoal action have not been widely investigated. One reason for this is the difficulty in culturing some protozoa (e.g., *Cryptosporidium*) under laboratory conditions. However, the different life stages (trophozoites and cysts) do provide a fascinating example of the problem

TABLE 1. Chemical structures and uses of biocides in antiseptics and disinfectants

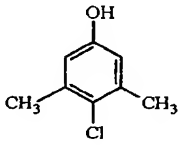
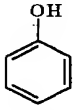
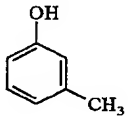
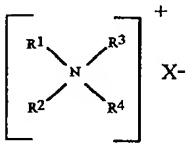
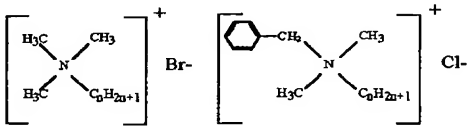
Alcohols	Ethanol	$CH_3 — CHOH$	Antisepsis
	Isopropanol		Disinfection
			Preservation
Aldehydes	Glutaraldehyde	$OH — CCH_2CH_2CH_2C — HO$	Disinfection
	Formaldehyde	$H — C — HO$	Sterilization
			Preservation
Anilides	General structure	$C_6H_5.NH.COR$	Antisepsis
	Triclocarban		
Biguanides	Chlorhexidine		Antisepsis
			Antiplaque agents
	Alexidine, polymeric biguanides		Preservation
			Disinfection
Bisphenols	Triclosan		Antisepsis
	Hexachlorophene		Antiplaque agents
			Deodorants
			Preservation
Diamidines	Propamidine		Antisepsis
			Preservation
	Dibromopropamidine		

Continued on following page

of how changes in cytology and physiology can modify responses to antiseptics and disinfectants. Khunkitti et al. (251–255) have explored this aspect by using indices of viability, leakage, uptake, and electron microscopy as experimental tools.

Some of these procedures can also be modified for studying effects on viruses and phages (e.g., uptake to whole cells and viral or phage components, effects on nucleic acids and proteins, and electron microscopy) (401). Viral targets are

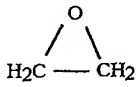
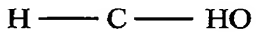
TABLE 1—*Continued*

Halogen-releasing agents	Chlorine compounds	⇳OCl-, HOCl, Cl_2	Disinfection Antisepsis
	Iodine compounds	⇳I_2	Cleaning
Halophenols	Chloroxylenol (PCMX)		Antisepsis Preservation
Heavy metal derivatives	Silver compounds	Ag	Preservation Antisepsis
	Mercury compounds	Hg	Disinfection
Peroxygens	Hydrogen peroxide	H_2O_2	Disinfection
	Ozone	O_3	Sterilization
	Peracetic acid	$CH_3.COOOH$	
Phenols and cresols	Phenol		Disinfection Preservation
	Cresol		
Quaternary ammonium compounds	General structure		Disinfection Antisepsis Preservation
	Cetrimide, benzalkonium chloride		Cleaning

Continued on following page

predominantly the viral envelope (if present), derived from the host cell cytoplasmic or nuclear membrane; the capsid, which is responsible for the shape of virus particles and for the protection of viral nucleic acid; and the viral genome. Release of an intact viral nucleic acid into the environment following capsid destruction is of potential concern since some nucleic acids are infective when liberated from the capsid (317), an aspect that must be considered in viral disinfection. Important considerations in viral inactivation are dealt with by Klein and Deforest (259) and Prince et al.

TABLE 1—*Continued*

Vapor phase	Ethylene oxide		Sterilization Disinfection
	Formaldehyde		
	Hydrogen peroxide	H_2O_2	

(384), while an earlier paper by Grossgebauer is highly recommended (189).

Alcohols

Although several alcohols have been shown to be effective antimicrobials, ethyl alcohol (ethanol, alcohol), isopropyl alcohol (isopropanol, propan-2-ol) and *n*-propanol (in particular in Europe) are the most widely used (337). Alcohols exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria (including mycobacteria), viruses, and fungi but are not sporicidal. They are, however, known to inhibit sporulation and spore germination (545), but this effect is reversible (513). Because of the lack of sporicidal activity, alcohols are not recommended for sterilization but are widely used for both hard-surface disinfection and skin antisepsis. Lower concentrations may also be used as preservatives and to potentiate the activity of other biocides. Many alcohol products include low levels of other biocides (in particular chlorhexidine), which remain on the skin following evaporation of the alcohol, or excipients (including emollients), which decrease the evaporation time of the alcohol and can significantly increase product efficacy (68). In general, isopropyl alcohol is considered slightly more efficacious against bacteria (95) and ethyl alcohol is more potent against viruses (259); however, this is dependent on the concentrations of both the active agent and the test microorganism. For example, isopropyl alcohol has greater lipophilic properties than ethyl alcohol and is less active against hydrophilic viruses (e.g., poliovirus) (259). Generally, the antimicrobial activity of alcohols is significantly lower at concentrations below 50% and is optimal in the 60 to 90% range.

Little is known about the specific mode of action of alcohols, but based on the increased efficacy in the presence of water, it is generally believed that they cause membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis (278, 337). This is supported by specific reports of denaturation of *Escherichia coli* dehydrogenases (499) and an increased lag phase in *Enterobacter aerogenes*, speculated to be due to inhibition of metabolism required for rapid cell division (101).

Aldehydes

Glutaraldehyde. Glutaraldehyde is an important dialdehyde that has found usage as a disinfectant and sterilant, in particular for low-temperature disinfection and sterilization of endoscopes and surgical equipment and as a fixative in electron

TABLE 2. Summary of mechanisms of antibacterial action of antiseptics and disinfectants

Target	Antiseptic or disinfectant	Mechanism of action
Cell envelope (cell wall, outer membrane)	Glutaraldehyde	Cross-linking of proteins
	EDTA, other permeabilizers	Gram-negative bacteria: removal of Mg^{2+}, release of some LPS
Cytoplasmic (inner) membrane	QACs	Generalized membrane damage involving phospholipid bilayers
	Chlorhexidine	Low concentrations affect membrane integrity, high concentrations cause congealing of cytoplasm
	Diamines	Induction of leakage of amino acids
	PHMB, alexidine	Phase separation and domain formation of membrane lipids
	Phenols	Leakage; some cause uncoupling
Cross-linking of macromolecules	Formaldehyde	Cross-linking of proteins, RNA, and DNA
	Glutaraldehyde	Cross-linking of proteins in cell envelope and elsewhere in the cell
DNA intercalation	Acridines	Intercalation of an acridine molecule between two layers of base pairs in DNA
Interaction with thiol groups	Silver compounds	Membrane-bound enzymes (interaction with thiol groups)
Effects on DNA	Halogens	Inhibition of DNA synthesis
	Hydrogen peroxide, silver ions	DNA strand breakage
Oxidizing agents	Halogens	Oxidation of thiol groups to disulfides, sulfoxides, or disulfoxides
	Peroxygens	Hydrogen peroxide: activity due to from formation of free hydroxy radicals ('OH), which oxidize thiol groups in enzymes and proteins; PAA: disruption of thiol groups in proteins and enzymes

TABLE 3. Mechanism of antimicrobial action of glutaraldehyde

Target microorganism	Glutaraldehyde action
Bacterial spores	Low concentrations inhibit germination; high concentrations are sporicidal, probably as a consequence of strong interaction with outer cell layers
Mycobacteria	Action unknown, but probably involves mycobacterial cell wall
Other nonsporulating bacteria	Strong association with outer layers of gram-positive and gram-negative bacteria; cross-linking of amino groups in protein; inhibition of transport processes into cell
Fungi	Fungal cell wall appears to be a primary target site, with postulated interaction with chitin
Viruses	Actual mechanisms unknown, but involve protein-DNA cross-links and capsid changes
Protozoa	Mechanism of action not known

icroscopy. Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi, and viruses, and a considerable amount of information is now available about the ways whereby these different organisms are inactivated (Tables 2 and 3). Earlier reviews of its mechanisms of action have been published (179, 182, 374, 482).

The first reports in 1964 and 1965 (182) demonstrated that glutaraldehyde possessed high antimicrobial activity. Subsequently, research was undertaken to evaluate the nature of its bactericidal (339–344, 450) and sporicidal (180, 181, 507, 508) action. These bactericidal studies demonstrated (374) a strong binding of glutaraldehyde to outer layers of organisms such as *E. coli* and *Staphylococcus aureus* (179, 212, 339–341, 343, 344), inhibition of transport in gram-negative bacteria (179), inhibition of dehydrogenase activity (343, 344) and of periplasmic enzymes (179), prevention of lysostaphin-induced lysis in *S. aureus* (453) and of sodium lauryl sulfate-induced lysis in *E. coli* (340, 344), inhibition of spheroplast and protoplast lysis in hypotonic media (340, 344), and inhibition of RNA, DNA, and protein synthesis (320). Strong interaction of glutaraldehyde with lysine and other amino acids has been demonstrated (450).

Clearly, the mechanism of action of glutaraldehyde involves a strong association with the outer layers of bacterial cells, specifically with unprotonated amines on the cell surface, possibly representing the reactive sites (65). Such an effect could explain its inhibitory action on transport and on enzyme systems, where access of substrate to enzyme is prohibited. Partial or entire removal of the cell wall in hypertonic medium, leading to the production of spheroplasts or protoplasts and the subsequent prevention of lysis by glutaraldehyde when these forms are diluted in a hypotonic environment, suggests an additional effect on the inner membrane, a finding substantiated by the fact that the dialdehyde prevents the selective release of some membrane-bound enzymes of *Micrococcus lysodeikticus* (138). Glutaraldehyde is more active at alkaline than at acidic pHs. As the external pH is altered from acidic to alkaline, more reactive sites will be formed at the cell surface, leading to a more rapid bactericidal effect. The cross-links thus obtained mean that the cell is then unable to undertake most, if not all, of its essential functions. Glutaraldehyde is also mycobactericidal. Unfortunately, no critical studies have as yet been undertaken to evaluate the nature of this action (419).

The bacterial spore presents several sites at which interaction with glutaraldehyde is possible, although interaction with a particular site does not necessarily mean that this is associated with spore inactivation. *E. coli*, *S. aureus*, and vegetative cells of *Bacillus subtilis* bind more glutaraldehyde than do resting spores of *B. subtilis* (377, 378); uptake of glutaraldehyde is greater during germination and outgrowth than with mature spores but still lower than with vegetative cells. Low concentrations of the dialdehyde (0.1%) inhibit germination, whereas much higher concentrations (2%) are sporicidal. The aldehyde, at both acidic and alkaline pHs, interacts strongly with the outer spore layers (508, 509); this interaction reduces the release of dipicolinic acid (DPA) from heated spores and the lysis induced by mercaptoethanol (or thioglycolate)-peroxide combinations. Low concentrations of both acidic and alkaline glutaraldehyde increase the surface hydrophobicity of spores, again indicating an effect at the outermost regions of the cell. It has been observed by various authors (182, 374, 376, 380) that the greater sporicidal activity of glutaraldehyde at alkaline pH is not reflected by differences in uptake; however, uptake per se reflects binding and not necessarily penetration into the spore. It is conceivable that acidic glutaraldehyde interacts with and remains at the cell surface whereas alkaline glutaraldehyde penetrates more deeply into the spore. This contention is at odds with the hypothesis of Bruch (65), who envisaged the acidic form penetrating the coat and reacting with the cortex while the alkaline form attacked the coat, thereby destroying the ability of the spore to function solely as a result of this surface phenomenon. There is, as yet, no evidence to support this theory. Novel glutaraldehyde formulations based on acidic rather than alkaline glutaraldehyde, which benefit from the greater inherent stability of the aldehyde at lower pH, have been produced. The improved sporicidal activity claimed for these products may be obtained by agents that potentiate the activity of the dialdehyde (414, 421).

During sporulation, the cell eventually becomes less susceptible to glutaraldehyde (see "Intrinsic resistance of bacterial spores"). By contrast, germinating and outgrowing cells reacquire sensitivity. Germination may be defined as an irreversible process in which there is a change of an activated spore from a dormant to a metabolically active state within a short period. Glutaraldehyde exerts an early effect on the germination process. L-Alanine is considered to act by binding to a specific receptor on the spore coat, and once spores are triggered to germinate, they are committed irreversibly to losing their dormant properties (491). Glutaraldehyde at high concentrations inhibits the uptake of L-[^{14}C]alanine by *B. subtilis* spores, albeit by an unknown mechanism (379, 414). Glutaraldehyde-treated spores retain their refractivity, having the same appearance under the phase-contrast microscope as normal, untreated spores even when the spores are subsequently incubated in germination medium. Glutaraldehyde is normally used as a 2% solution to achieve a sporicidal effect (16, 316); low concentrations (<0.1%) prevent phase darkening of spores and also prevent the decrease in optical density associated with a late event in germination. By contrast, higher concentrations (0.1 to 1%) significantly reduce the uptake of L-alanine, possibly as a result of a sealing effect of the aldehyde on the cell surface. Mechanisms involved in the revival of glutaraldehyde-treated spores are discussed below (see "Intrinsic resistance of bacterial spores").

There are no recent studies of the mechanisms of fungicidal action of glutaraldehyde. Earlier work had suggested that the fungal cell wall was a major target site (179, 182, 352), especially the major wall component, chitin, which is analogous to the peptidoglycan found in bacterial cell walls.

Glutaraldehyde is a potent virucidal agent (143, 260). It reduces the activity of hepatitis B surface antigen (HBsAg) and especially hepatitis B core antigen ([HBcAg] in hepatitis B virus [HBV]) (3) and interacts with lysine residues on the surface of hepatitis A virus (HAV) (362). Low concentrations

(<0.1%) of alkaline glutaraldehyde are effective against purified poliovirus, whereas poliovirus RNA is highly resistant to aldehyde concentrations up to 1% at pH 7.2 and is only slowly inactivated at pH 8.3 (21). In other words, the complete poliovirus particle is much more sensitive than poliovirus RNA. In light of this, it has been inferred that glutaraldehyde-induced loss of infectivity is associated with capsid changes (21). Glutaraldehyde at the low concentrations of 0.05 and 0.005% interacts with the capsid proteins of poliovirus and echovirus, respectively; the differences in sensitivity probably reflect major structural variations in the two viruses (75).

Bacteriophages were recently studied to obtain information about mechanisms of virucidal action (298–304, 306, 307). Many glutaraldehyde-treated *P. aeruginosa* F116 phage particles had empty heads, implying that the phage genome had been ejected. The aldehyde was possibly bound to F116 double-stranded DNA but without affecting the molecule; glutaraldehyde also interacted with phage F116 proteins, which were postulated to be involved in the ejection of the nucleic acid. Concentrations of glutaraldehyde greater than 0.1 to 0.25% significantly affected the transduction of this phage; the transduction process was more sensitive to the aldehyde than was the phage itself. Glutaraldehyde and other aldehydes were tested for their ability to form protein-DNA cross-links in simian virus 40 (SV40); aldehydes (i.e., glyoxal, furfural, prionaldehyde, acetaldehyde, and benzylaldehyde) without detectable cross-linking ability had no effect on SV40 DNA synthesis, whereas acrolein, glutaraldehyde, and formaldehyde, which formed such cross-links (144, 271, 297), inhibited DNA synthesis (369).

Formaldehyde. Formaldehyde (methanal, CH_2O) is a monoaldehyde that exists as a freely water-soluble gas. Formaldehyde solution (formalin) is an aqueous solution containing *ca.* 34 to 38% (wt/wt) CH_2O with methanol to delay polymerization. Its clinical use is generally as a disinfectant and sterilant in liquid or in combination with low-temperature steam. Formaldehyde is bactericidal, sporicidal, and virucidal, but it works more slowly than glutaraldehyde (374, 482).

Formaldehyde is an extremely reactive chemical (374, 442) that interacts with protein (156, 157), DNA (155), and RNA (155) in vitro. It has long been considered to be sporicidal by virtue of its ability to penetrate into the interior of bacterial spores (500). The interaction with protein results from a combination with the primary amide as well as with the amino groups, although phenol groups bind little formaldehyde (155). It has been proposed that formaldehyde acts as a mutagenic agent (291) and as an alkylating agent by reaction with carboxyl, sulfhydryl, and hydroxyl groups (371). Formaldehyde also reacts extensively with nucleic acid (489) (e.g., the DNA of bacteriophage T2) (190). As pointed out above, it forms protein-DNA cross-links in SV40, thereby inhibiting DNA synthesis (369). Low concentrations of formaldehyde are sporostatic and inhibit germination (512). Formaldehyde alters HBsAg and HBcAg of HBV (3).

It is difficult to pinpoint accurately the mechanism(s) responsible for formaldehyde-induced microbial inactivation. Clearly, its interactive, and cross-linking properties must play a considerable role in this activity. Most of the other aldehydes (glutaraldehyde, glyoxyl, succinaldehyde, and *o*-phthalaldehyde [OPA]) that have sporicidal activity are dialdehydes (and of these, glyoxyl and succinaldehyde are weakly active). The distance between the two aldehyde groups in glutaraldehyde (and possibly in OPA) may be optimal for interaction of these-CHO groups in nucleic acids and especially in proteins and enzymes (428).

Formaldehyde-releasing agents. Several formaldehyde-releasing agents have been used in the treatment of peritonitis (226, 273). They include noxythiolin (oxymethylenethiourea), tauroline (a condensate of two molecules of the aminosulponic acid taurine with three molecules of formaldehyde), hexamine (hexamethylenetetramine, methenamine), the resins melamine and urea formaldehydes, and imidazolone derivatives such as dantoin. All of these agents are claimed to be microbicidal on account of the release of formaldehyde. However, because the antibacterial activity of taurolin is greater than that of free formaldehyde, the activity of taurolin is not entirely the result of formaldehyde action (247).

TABLE 4. Mechanisms of antimicrobial action of chlorhexidine

Type of microorganism	Chlorhexidine action
Bacterial spores	Not sporicidal but prevents development of spores; inhibits spore outgrowth but not germination
Mycobacteria	Mycobacteristatic (mechanism unknown) but not mycobactericidal
Other nonsporulating bacteria	Membrane-active agent, causing protoplast and spheroplast lysis; high concentrations cause precipitation of proteins and nucleic acids
Yeasts	Membrane-active agent, causing protoplast lysis and intracellular leakage; high concentrations cause intracellular coagulation
Viruses	Low activity against many viruses; lipid-enveloped viruses more sensitive than nonenveloped viruses; effect possibly on viral envelope, perhaps the lipid moieties
Protozoa	Recent studies against *A. castellanii* demonstrate membrane activity (leakage) toward trophozoites, less toward cysts

***o*-Phthalaldehyde.** OPA is a new type of disinfectant that is claimed to have potent bactericidal and sporicidal activity and has been suggested as a replacement for glutaraldehyde in endoscope disinfection (7). OPA is an aromatic compound with two aldehyde groups. To date, the mechanism of its antimicrobial action has been little studied, but preliminary evidence (526) suggests an action similar to that of glutaraldehyde. Further investigations are needed to corroborate this opinion.

Anilides

The anilides have been investigated primarily for use as antiseptics, but they are rarely used in the clinic. Triclocarban (TCC; 3,4,4′-triclorocarbanilide) is the most extensively studied in this series and is used mostly in consumer soaps and deodorants. TCC is particularly active against gram-positive bacteria but significantly less active against gram-negative bacteria and fungi (30) and lacks appreciable substantivity (persistency) for the skin (37). The anilides are thought to act by adsorbing to and destroying the semipermeable character of the cytoplasmic membrane, leading to cell death (194).

Biguanides

Chlorhexidine. Chlorhexidine is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products but also as a disinfectant and preservative. This is due in particular to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific (167, 403). Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (430). A considerable amount of research has been undertaken on the mechanism of the antimicrobial action of this important bisbiguanide (389) (Tables 2 and 4), although most of the attention has been devoted to the way in which it

inactivates nonsporulating bacteria (215, 428, 430, 431, 451). Nevertheless, sufficient data are now available to examine its sporostatic and mycobacteriostatic action, its effects on yeasts and protozoa, and its antiviral activity.

Chlorhexidine is a bactericidal agent (120, 215). Its interaction and uptake by bacteria were studied initially by Hugo et al. (222–224), who found that the uptake of chlorhexidine by *E. coli* and *S. aureus* was very rapid and depended on the chlorhexidine concentration and pH. More recently, by using [^{14}C]chlorhexidine gluconate, the uptake by bacteria (145) and yeasts (204) was shown to be extremely rapid, with a maximum effect occurring within 20 s. Damage to the outer cell layers takes place (139) but is insufficient to induce lysis or cell death. The agent then crosses the cell wall or outer membrane, presumably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane or the yeast plasma membrane. In yeasts, chlorhexidine "partitions" into the cell wall, plasma membrane, and cytoplasm of cells (205). Damage to the delicate semipermeable membrane is followed by leakage of intracellular constituents, which can be measured by appropriate techniques. Leakage is not per se responsible for cellular inactivation but is a consequence of cell death (445). High concentrations of chlorhexidine cause coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage (222–224, 290), so that there is a biphasic effect on membrane permeability. An initial high rate of leakage rises as the concentration of chlorhexidine increases, but leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol.

Chlorhexidine was claimed by Harold et al. (199) to be an inhibitor of both membrane-bound and soluble ATPase as well as of net K^+ uptake in *Enterococcus faecalis*. However, only high biguanide concentrations inhibit membrane-bound ATPase (83), which suggests that the enzyme is not a primary target for chlorhexidine action. Although chlorhexidine collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its lethal effects (24, 272).

The effects of chlorhexidine on yeast cells are probably similar to those previously described for bacteria (204–207). Chlorhexidine has a biphasic effect on protoplast lysis, with reduced lysis at higher biguanide concentrations. Furthermore, in whole cells, the yeast cell wall may have some effect in limiting the uptake of the biguanide (208). The findings presented here and elsewhere (47, 136, 137, 527) demonstrate an effect on the fungal plasma membrane but with significant actions elsewhere in the cell (47). Increasing concentrations of chlorhexidine (up to 25 μg/ml) induce progressive lysis of *Saccharomyces cerevisiae* protoplasts, but higher biguanide concentrations result in reduced lysis (205).

Work to date suggests that chlorhexidine has a similar effect on the trophozoites of *Acanthameoba castellanii*, with the cysts being less sensitive (251–255). Furr (163) reviewed the effects of chlorhexidine and other biocides on *Acanthameoba* and showed that membrane damage in these protozoa is a significant factor in their inactivation.

Mycobacteria are generally highly resistant to chlorhexidine (419). Little is known about the uptake of chlorhexidine (and other antiseptics and disinfectants) by mycobacteria and on the biochemical changes that occur in the treated cells. Since the MICs for some mycobacteria are on the order of those for chlorhexidine-sensitive, gram-positive cocci (48), the inhibitory effects of chlorhexidine on mycobacteria may not be dissimilar to those on susceptible bacteria. *Mycobacterium avium-intracellulare* is considerably more resistant than other mycobacteria (48).

Chlorhexidine is not sporicidal (discussed in "Mechanisms of resistance"). Even high concentrations of the bisbiguanide do not affect the viability of *Bacillus* spores at ambient temperatures (473, 474), although a marked sporicidal effect is achieved at elevated temperatures (475). Presumably, sufficient changes occur in the spore structure to permit an increased uptake of the biguanide, although this has yet to be shown experimentally. Little is known about the uptake of chlorhexidine by bacterial spores, although coatless forms take up more of the compound than do "normal" spores (474).

Chlorhexidine has little effect on the germination of bacterial spores (414, 422, 432, 447) but inhibits outgrowth (447). The reason for its lack of effect on the former process but its significant activity against the latter is unclear. It could, however, be reflected in the relative uptake of chlorhexidine, since germinating cells take up much less of the bisbiguanide than do outgrowing forms (474). Binding sites could thus be reduced in number or masked in germinating cells.

The antiviral activity of chlorhexidine is variable. Studies with different types of bacteriophages have shown that chlorhexidine has no effect on MS2 or K coliphages (300). High concentrations also failed to inactivate *Pseudomonas aeruginosa* phage F116 and had no effect on phage DNA within the capsid or on phage proteins (301); the transduction process was more sensitive to chlorhexidine and other biocides than was the phage itself. This substantiated an earlier finding (306) that chlorhexidine bound poorly to F116 particles. Chlorhexidine is not always considered a particularly effective antiviral agent, and its activity is restricted to the lipid-enveloped viruses (361). Chlorhexidine does not inactivate nonenveloped viruses such as rotavirus (485), HAV (315), or poliovirus (34). Its activity was found by Ranganathan (389) to be restricted to the nucleic acid core or the outer coat, although it is likely that the latter would be a more important target site.

Alexidine. Alexidine differs chemically from chlorhexidine in possessing ethylhexyl end groups. Alexidine is more rapidly bactericidal and produces a significantly faster alteration in bactericidal permeability (79, 80). Studies with mixed-lipid and pure phospholipid vesicles demonstrate that, unlike chlorhexidine, alexidine produces lipid phase separation and domain formation (Table 2). It has been proposed (80) that the nature of the ethylhexyl end group in alexidine, as opposed to the chlorophenol one in chlorhexidine, might influence the ability of a biguanide to produce lipid domains in the cytoplasmic membrane.

Polymeric biguanides. Vantocil is a heterodisperse mixture of polyhexamethylene biguanides (PHMB) with a molecular weight of approximately 3,000. Polymeric biguanides have found use as general disinfecting agents in the food industry and, very successfully, for the disinfection of swimming pools. Vantocil is active against gram-positive and gram-negative bacteria, although *P. aeruginosa* and *Proteus vulgaris* are less sensitive. Vantocil is not sporicidal. PHMB is a membrane-active agent that also impairs the integrity of the outer membrane of gram-negative bacteria, although the membrane may also act as a permeability barrier (64, 172). Activity of PHMB increases on a weight basis with increasing levels of polymerization, which has been linked to enhanced inner membrane perturbation (173, 174).

Unlike chlorhexidine but similar to alexidine (Table 2), PHMB causes domain formation of the acidic phospholipids of the cytoplasmic membrane (61–64, 172, 173, 227). Permeability changes ensue, and there is believed to be an altered function of some membrane-associated enzymes. The proposed sequence of events during its interaction with the cell envelope of *E. coli* is as follows: (i) there is rapid attraction of

PHMB toward the negatively charged bacterial cell surface, with strong and specific adsorption to phosphate-containing compounds; (ii) the integrity of the outer membrane is impaired, and PHMB is attracted to the inner membrane; (iii) binding of PHMB to phospholipids occurs, with an increase in inner membrane permeability (K^+ loss) accompanied by bacteriostasis; and (iv) complete loss of membrane function follows, with precipitation of intracellular constituents and a bactericidal effect.

Diamidines

The diamidines are characterized chemically as described in Table 1. The isethionate salts of two compounds, propamidine (4,4-diaminodiphenoxypropane) and dibromopropamidine (2,2-dibromo-4,4-diamidinodiphenoxypropane), have been used as antibacterial agents. Their antibacterial properties and uses were reviewed by Hugo (213) and Hugo and Russell (226). Clinically, diamidines are used for the topical treatment of wounds.

The exact mechanism of action of diamidines is unknown, but they have been shown to inhibit oxygen uptake and induce leakage of amino acids (Table 2), as would be expected if they are considered as cationic surface-active agents. Damage to the cell surface of *P. aeruginosa* and *Enterobacter cloacae* has been described (400).

Halogen-Releasing Agents

Chlorine- and iodine-based compounds are the most significant microbicidal halogens used in the clinic and have been traditionally used for both antiseptic and disinfectant purposes.

Chlorine-releasing agents. Excellent reviews that deal with the chemical, physical, and microbiological properties of chlorine-releasing agents (CRAs) are available (42, 130). The most important types of CRAs are sodium hypochlorite, chlorine dioxide, and the *N*-chloro compounds such as sodium dichloroisocyanurate (NaDCC), with chloramine-T being used to some extent. Sodium hypochlorite solutions are widely used for hard-surface disinfection (household bleach) and can be used for disinfecting spillages of blood containing human immunodeficiency virus or HBV. NaDCC can also be used for this purpose and has the advantages of providing a higher concentration of available chlorine and being less susceptible to inactivation by organic matter. In water, sodium hypochlorite ionizes to produce Na^+ and the hypochlorite ion, OCl^-, which establishes an equilibrium with hypochlorous acid, HOCl (42). Between pH 4 and 7, chlorine exists predominantly as HClO, the active moiety, whereas above pH9, OCl^- predominates. Although CRAs have been predominantly used as hard-surface disinfectants, novel acidified sodium chlorite (a two-component system of sodium chlorite and mandelic acid) has been described as an effective antiseptic (248).

Surprisingly, despite being widely studied, the actual mechanism of action of CRAs is not fully known (Table 2). CRAs are highly active oxidizing agents and thereby destroy the cellular activity of proteins (42); potentiation of oxidation may occur at low pH, where the activity of CRAs is maximal, although increased penetration of outer cell layers may be achieved with CRAs in the unionized state. Hypochlorous acid has long been considered the active moiety responsible for bacterial inactivation by CRAs, the OCl^- ion having a minute effect compared to undissolved HOCl (130). This correlates with the observation that CRA activity is greatest when the percentage of undissolved HOCl is highest. This concept applies to hypochlorites, NaDCC, and chloramine-T.

Deleterious effects of CRAs on bacterial DNA that involve the formation of chlorinated derivatives of nucleotide bases have been described (115, 128, 477). Hypochlorous acid has also been found to disrupt oxidative phosphorylation (26) and other membrane-associated activity (70). In a particularly interesting paper, McKenna and Davies (321) described the inhibition of bacterial growth by hypochlorous acid. At 50 μM (2.6 ppm), HOCl completely inhibited the growth of *E. coli* within 5 min, and DNA synthesis was inhibited by 96% but protein synthesis was inhibited by only 10 to 30%. Because concentrations below 5 mM (260 ppm) did not induce bacterial membrane disruption or extensive protein degradation, it was inferred that DNA synthesis was the sensitive target. In contrast, chlorine dioxide inhibited bacterial protein synthesis (33).

CRAs at higher concentrations are sporicidal (44, 421, 431); this depends on the pH and concentration of available chlorine (408, 412). During treatment, the spores lose refractivity, the spore coat separates from the cortex, and lysis occurs (268). In addition, a number of studies have concluded that CRA-treated spores exhibit increased permeability of the spore coat (131, 268, 412).

CRAs also possess virucidal activity (34, 46, 116, 315, 394, 407, 467, 485, 486). Olivieri et al. (359) showed that chlorine inactivated naked f2 RNA at the same rate as RNA in intact phage, whereas f2 capsid proteins could still adsorb to the host. Taylor and Butler (504) found that the RNA of poliovirus type 1 was degraded into fragments by chlorine but that poliovirus inactivation preceded any severe morphological changes. By contrast, Floyd et al. (149) and O'Brien and Newman (357) demonstrated that the capsid of poliovirus type 1 was broken down. Clearly, further studies are needed to explain the antiviral action of CRAs.

Iodine and iodophors. Although less reactive than chlorine, iodine is rapidly bactericidal, fungicidal, tuberculocidal, virucidal, and sporicidal (184). Although aqueous or alcoholic (tincture) solutions of iodine have been used for 150 years as antiseptics, they are associated with irritation and excessive staining. In addition, aqueous solutions are generally unstable; in solution, at least seven iodine species are present in a complex equilibrium, with molecular iodine (I_2) being primarily responsible for antimictrobial efficacy (184). These problems were overcome by the development of iodophors ("iodine carriers" or "iodine-releasing agents"); the most widely used are povidone-iodine and poloxamer-iodine in both antiseptics and disinfectants. Iodophors are complexes of iodine and a solubilizing agent or carrier, which acts as a reservoir of the active "free" iodine (184). Although germicidal activity is maintained, iodophors are considered less active against certain fungi and spores than are tinctures (454).

Similar to chlorine, the antimicrobial action of iodine is rapid, even at low concentrations, but the exact mode of action is unknown. Iodine rapidly penetrates into microorganisms (76) and attacks key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine [184, 267]), nucleotides, and fatty acids (15, 184), which culminates in cell death (184). Less is known about the antiviral action of iodine, but nonlipid viruses and parvoviruses are less sensitive than lipid enveloped viruses (384). Similarly to bacteria, it is likely that iodine attacks the surface proteins of enveloped viruses, but they may also destabilize membrane fatty acids by reacting with unsaturated carbon bonds (486).

Silver Compounds

In one form or another, silver and its compounds have long been used as antimicrobial agents (55, 443). The most important silver compound currently in use is silver sulfadiazine

(AgSD), although silver metal, silver acetate, silver nitrate, and silver protein, all of which have antimicrobial properties, are listed in *Martindale, The Extra Pharmacopoeia* (312). In recent years, silver compounds have been used to prevent the infection of burns and some eye infections and to destroy warts.

Silver nitrate. The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfydryl, –SH) groups (32, 49, 161, 164), although other target sites remain a possibility (397, 509). Liau et al (287) demonstrated that amino acids such as cysteine and other compounds such as sodium thioglycolate containing thiol groups neutralized the activity of silver nitrate against *P. aeruginosa*. By contrast, amino acids containing disulfide (SS) bonds, non-sulfur-containing amino acids, and sulfur-containing compounds such as cystathione, cysteic acid, L-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag^+ activity. These and other findings imply that interaction of Ag^+ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved. Hydrogen bonding, the effects of hydrogen bond-breaking agents, and the specificity of Ag^+ for thiol groups were discussed in greater detail by Russell and Hugo (443) (Table 2). Virucidal properties might also be explained by binding to –SH groups (510).

Lukens (292) proposed that silver salts and other heavy metals such as copper act by binding to key functional groups of fungal enzymes. Ag^+ causes the release of K^+ ions from microorganisms; the microbial plasma or cytoplasmic membrane, with which is associated many important enzymes, is an important target site for Ag^+ activity (161, 329, 392, 470).

In addition to its effects on enzymes, Ag^+ produces other changes in microorganisms. Silver nitrate causes marked inhibition of growth of *Cryptococcus neoformans* and is deposited in the vacuole and cell wall as granules (60). Ag^+ inhibits cell division and damages the cell envelope and contents of *P. aeruginosa* (398). Bacterial cells increase in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibit structural abnormalities, although without any blebs (protuberances) (398). Finally, the Ag^+ ion interacts with nucleic acids (543); it interacts preferentially with the bases in DNA rather than with the phosphate groups, although the significance of this in terms of its lethal action is unclear (231, 387, 510, 547).

Silver sulfadiazine. AgSD is essentially a combination of two antibacterial agents, Ag^+ and sulfadiazine (SD). The question whether the antibacterial effect of AgSD arises predominantly from only one of the compounds or via a synergistic interaction has been posed repeatedly. AgSD has a broad spectrum of activity and, unlike silver nitrate, produces surface and membrane blebs in susceptible (but not resistant) bacteria (96). AgSD binds to cell components, including DNA (332, 404). Based on a chemical analysis, Fox (153) proposed a polymeric structure of AgSD composed of six silver atoms bonding to six SD molecules by linkage of the silver atoms to the nitrogens of the SD pyrimidine ring. Bacterial inhibition would then presumably be achieved when silver binds to sufficient base pairs in the DNA helix, thereby inhibiting transcription. Similarly, its antiphage properties have been ascribed to the fact that AgSD binds to phage DNA (154, 388). Clearly, the precise mechanism of action of AgSD has yet to be solved.

Peroxygens

Hydrogen peroxide. Hydrogen peroxide (H_2O_2) is a widely used biocide for disinfection, sterilization, and antisepsis. It is a clear, colorless liquid that is commercially available in a variety of concentrations ranging from 3 to 90%. H_2O_2 is considered environmentally friendly, because it can rapidly degrade into the innocuous products water and oxygen. Although pure solutions are generally stable, most contain stabilizers to prevent decomposition. H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores (38). In general, greater activity is seen against gram-positive than gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations. Higher concentrations of H_2O_2 (10 to 30%) and longer contact times are required for sporicidal activity (416), although this activity is significantly increased in the gaseous phase. H_2O_2 acts as an oxidant by producing hydroxyl free radicals ($^{\bullet}OH$) which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulfhydryl groups and double bonds are particularly targeted (38).

Peracetic acid. Peracetic acid (PAA) (CH_3COOOH) is considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (<0.3%) (38). PAA also decomposes to safe by-products (acetic acid and oxygen) but has the added advantages of being free from decomposition by peroxidases, unlike H_2O_2, and remaining active in the presence of organic loads (283, 308). Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (100, 308).

Similar to H_2O_2, PAA probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (–SH) and sulfur (S–S) bonds (22, 38).

Phenols

Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound. It has been known for many years (215) that, although they have often been referred to as "general protoplasmic poisons," they have membrane-active properties which also contribute to their overall activity (120) (Table 2).

Phenol induces progressive leakage of intracellular constituents, including the release of K^+, the first index of membrane damage (273), and of radioactivity from ^{14}C-labeled *E. coli* (242, 265). Pulvertaft and Lumb (386) demonstrated that low concentrations of phenols (0.032%, 320 μg/ml) and other (nonphenolic) agents lysed rapidly growing cultures of *E. coli*, staphylococci, and streptococci and concluded that autolytic enzymes were not involved. Srivastava and Thompson (487, 488) proposed that phenol acts only at the point of separation of pairs of daughter cells, with young bacterial cells being more sensitive than older cells to phenol.

Hugo and Bloomfield (216, 217) showed with the chlorinated bis-phenol fenticlor that there was a close relationship between bactericidal activity and leakage of 260-nm-absorbing material (leakage being induced only by bactericidal concentrations). Fentichlor affected the metabolic activities of *S. aureus* and *E. coli* (217) and produced a selective increase in permeability to protons with a consequent dissipation of the proton motive force (PMF) and an uncoupling of oxidative phosphorylation (41). Chlorocresol has a similar action (124). Coagulation of cytoplasmic constituents at higher phenol concentrations, which causes irreversible cellular damage, has been described by Hugo (215).

The phenolics possess antifungal and antiviral properties. Their antifungal action probably involves damage to the plas-

ma membrane (436), resulting in leakage of intracellular constituents. Phenol does not affect the transduction of *P. aeruginosa* PAO by bacteriophage F116 (301), has no effect on phage DNA within the capsid, and has little effect on several of the phage band proteins unless treatments of 20 min or longer are used (303, 304).

Bis-Phenols

The bis-phenols are hydroxy-halogenated derivatives of two phenolic groups connected by various bridges (191, 446). In general, they exhibit broad-spectrum efficacy but have little activity against *P. aeruginosa* and molds and are sporostatic toward bacterial spores. Triclosan and hexachlorophane are the most widely used biocides in this group, especially in antiseptic soaps and hand rinses. Both compounds have been shown to have cumulative and persistent effects on the skin (313).

Triclosan. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether; Irgasan DP 300) exhibits particular activity against gram-positive bacteria (469, 521). Its efficacy against gram-negative bacteria and yeasts can be significantly enhanced by formulation effects. For example, triclosan in combination with EDTA caused increased permeability of the outer membrane (282). Reports have also suggested that in addition to its antibacterial properties, triclosan may have anti-inflammatory activity (25, 522). The specific mode of action of triclosan is unknown, but it has been suggested that the primary effects are on the cytoplasmic membrane. In studies with *E. coli*, triclosan at subinhibitory concentrations inhibited the uptake of essential nutrients, while higher, bactericidal concentrations resulted in the rapid release of cellular components and cell death (393). Studies with a divalent-ion-dependent *E. coli* triclosan mutant for which the triclosan MIC was 10-fold greater than that for a wild-type strain showed no significant differences in total envelope protein profiles but did show significant differences in envelope fatty acids (370). Specifically, a prominent 14:1 fatty acid was absent in the resistant strain, and there were minor differences in other fatty acid species. It was proposed that divalent ions and fatty acids may adsorb and limit the permeability of triclosan to its site of action (370). Minor changes in fatty acid profiles were recently found in both *E. coli* and *S. aureus* strains for which the triclosan MICs were elevated; however, the MBCs were not affected, suggesting, as for other phenols, that the cumulative effects on multiple targets contribute to the bactericidal activity (318, 319).

Hexachlorophene. Hexachlorophene (hexachlorophane; 2,2′-dihydroxy-3,5,6,3′,5′,6′-hexachlorodiphenylmethane) is another bis-phenol whose mode of action has been extensively studied. The primary action of hexachlorophene, based on studies with *Bacillus megatherium*, is to inhibit the membrane-bound part of the electron transport chain, and the other effects noted above are secondary ones that occur only at high concentrations (92, 158, 241, 481). It induces leakage, causes protoplast lysis, and inhibits respiration. The threshold concentration for the bactericidal activity of hexachlorphene is 10 μg/ml (dry weight), but peak leakage occurs at concentrations higher than 50 μg/ml and cytological changes occur above 30 μg/ml. Furthermore, hexachlorophene is bactericidal at 0°C despite causing little leakage at this temperature. Despite the broad-spectrum efficacy of hexachlorophene, concerns about toxicity (256), in particular in neonates, have meant that its use in antiseptic products has been limited.

Halophenols

Chloroxylenol (4-chloro-3,5-dimethylphenol; *p*-chloro-*m*-xylenol) is the key halophenol used in antiseptic or disinfectant formulations (66). Chloroxylenol is bactericidal, but *P. aeruginosa* and many molds are highly resistant (66, 432). Surprisingly, its mechanism of action has been little studied despite its widespread use over many years. Because of its phenolic nature, it would be expected to have an effect on microbial membranes.

Quaternary Ammonium Compounds

Surface-active agents (surfactants) have two regions in their molecular structures, one a hydrocarbon, water-repellent (hydrophobic) group and the other a water-attracting (hydrophilic or polar) group. Depending on the basis of the charge or absence of ionization of the hydrophilic group, surfactants are classified into cationic, anionic, nonionic, and ampholytic (amphoteric) compounds. Of these, the cationic agents, as exemplified by quaternary ammonium compounds (QACs), are the most useful antiseptics and disinfectants (160). They are sometimes known as cationic detergents. QACs have been used for a variety of clinical purposes (e.g., preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces). In addition to having antimicrobial properties, QACs are also excellent for hard-surface cleaning and deodorization.

It has been known for many years that QACs are membrane-active agents (221) (Table 2) (i.e., with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts) (215). Salton (460) proposed the following sequence of events with microorganisms exposed to cationic agents: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (120).

Useful information about the selectivity of membrane action can be obtained by studying the effects of biocides on protoplasts and spheroplasts suspended in various solutes. QACs cause lysis of spheroplasts and protoplasts suspended in sucrose (107, 215, 243, 428). The cationic agents react with phospholipid components in the cytoplasmic membrane (69), thereby producing membrane distortion and protoplast lysis under osmotic stress. Isolated membranes do not undergo disaggregation on exposure to QACs, because the membrane distortion is not sufficiently drastic. The non-QAC agents TCC and trichlorosalicylanide have specific effects: TCC induces protoplast lysis in ammonium chloride by increasing Cl^- permeability, whereas trichlorosalicylanide induces lysis in ammonium nitrate by increasing NO_3^- permeability (428). In contrast, QACs (and chlorhexidine) induce lysis of protoplasts or spheroplasts suspended in various solutes because they effect generalized, rather than specific, membrane damage.

The bacterial cytoplasmic membrane provides the mechanism whereby metabolism is linked to solute transport, flagellar movement, and the generation of ATP. Protons are extruded to the exterior of the bacterial cell during metabolism. The combined potential (concentration or osmotic effect of the proton and its electropositivity) is the PMF, which drives these ancillary activities (428). The QAC cetrimide was found (121) to have an effect on the PMF in *S. aureus*. At its bacteriostatic concentration, cetrimide caused the discharge of the pH component of the PMF and also produced the maximum amount of 260-nm-absorbing material.

QACs are also believed to damage the outer membrane of gram-negative bacteria, thereby promoting their own uptake. This aspect of QACs is considered below (see "Intrinsic resistance of gram-negative bacteria").

The QAC cetylpyridium chloride (CPC) induces the leakage of K^+ and pentose material from the yeast *S. cerevisiae* and induces protoplast lysis as well as interacting with crude cell sap (205). Unlike chlorhexidine, however, no biphasic effect on protoplast lysis was observed. The initial toxic effect of QACs on yeast cells is a disorganization of the plasma membranes, with organized lipid structures in the membranes (and in lipid bilayers) being disrupted.

QACs are sporostatic; they inhibit the outgrowth of spores (the development of a vegetative cell from a germinated spore) but not the actual germination processes (development from dormancy to a metabolically active state), albeit by an unknown mechanism (414). Likewise, the QACs are not mycobactericidal but have a mycobacteriostatic action, although the actual effects on mycobacteria have been little studied (419).

The QACs have an effect on lipid, enveloped (including human immunodeficiency virus and HBV) but not nonenveloped viruses (394, 485, 486). QAC-based products induced disintegration and morphological changes of human HBV, resulting in loss of infectivity (382). In studies with different phages (298–301, 303–305, 307), CPC significantly inhibited transduction by bacteriophage F116 and inactivated the phage particles. Furthermore, CPC altered the protein bands of F116 but did not affect the phage DNA within the capsid.

Vapor-Phase Sterilants

Many heat-sensitive medical devices and surgical supplies can be effectively sterilized by liquid sterilants (in particular glutaraldehyde, PAA, and hydrogen peroxide) or by vapor-phase sterilization systems (Table 1). The most widely used active agents in these "cold" systems are ethylene oxide, formaldehyde and, more recently developed, hydrogen peroxide and PAA. Ethylene oxide and formaldehyde are both broad-spectrum alkylating agents. However, their activity is dependent on active concentration, temperature, duration of exposure, and relative humidity (87). As alkylating agents, they attack proteins, nucleic acids, and other organic compounds; both are particularly reactive with sulfhydryl and other enzyme-reactive groups. Ethylene oxide gas has the disadvantages of being mutagenic and explosive but is not generally harsh on sensitive equipment, and toxic residuals from the sterilization procedure can be routinely eliminated by correct aeration. Formaldehyde gas is similar and has the added advantage of being nonexplosive but is not widely used in health care. Vapor-phase hydrogen peroxide and PAA are considered more active (as oxidants) at lower concentrations than in the liquid form (334). Both active agents are used in combination with gas plasma in low-temperature sterilization systems (314). Their main advantages over other vapor-phase systems include low toxicity, rapid action, and activity at lower temperature; the disadvantages include limited penetrability and applications.

MECHANISMS OF RESISTANCE

Introduction

As stated above, different types of microorganisms vary in their response to antiseptics and disinfectants. This is hardly surprising in view of their different cellular structure, composition, and physiology. Traditionally, microbial susceptibility to antiseptics and disinfectants has been classified based on these differences; with recent work, this classification can be further extended (Fig. 1). Because different types of organisms react differently, it is convenient to consider bacteria, fungi, viruses, protozoa, and prions separately.

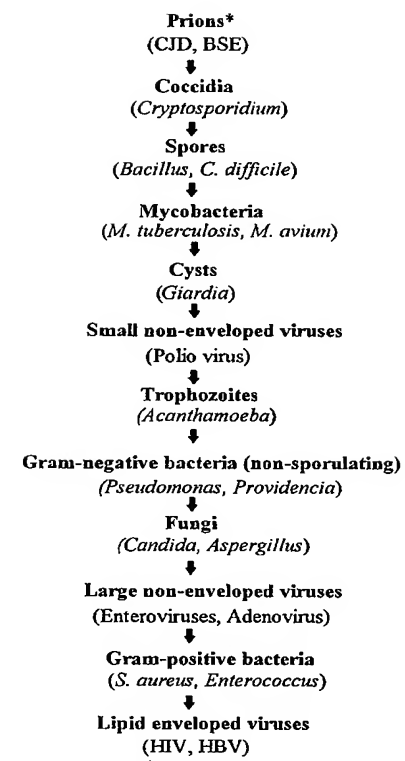


FIG. 1. Descending order of resistance to antiseptics and disinfectants. The asterisk indicates that the conclusions are not yet universally agreed upon.

Bacterial Resistance to Antiseptics and Disinfectants

In recent years, considerable progress has been made in understanding more fully the responses of different types of bacteria (mycobacteria, nonsporulating bacteria, and bacterial spores) to antibacterial agents (43, 84, 414, 415, 419, 422, 496). As a result, resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes). Intrinsic resistance is demonstrated by gram-negative bacteria, bacterial spores, mycobacteria, and, under certain conditions, staphylococci (Table 5). Acquired, plasmid-mediated resistance is most widely associated with mercury compounds and other metallic salts. In recent years, acquired resistance to certain other types of biocides has been observed, notably in staphylococci.

Intrinsic Bacterial Resistance Mechanisms

For an antiseptic or disinfectant molecule to reach its target site, the outer layers of a cell must be crossed. The nature and composition of these layers depend on the organism type and may act as a permeability barrier, in which there may be a reduced uptake (422, 428). Alternatively but less commonly, constitutively synthesized enzymes may bring about degradation of a compound (43, 214, 358). Intrinsic (innate) resistance

TABLE 5. Intrinsic resistance mechanisms in bacteria to antiseptics and disinfectants

Type of resistance	Example(s)	Mechanism of resistance
Impermeability		
Gram-negative bacteria	QACs, triclosan, diamines	Barrier presented by outer membrane may prevent uptake of antiseptic or disinfectant; glycocalyx may also be involved
Mycobacteria	Chlorhexidine, QACs Glutaraldehyde	Waxy cell wall prevents adequate biocide entry Reason for high resistance of some strains of *M. chelonae*(?)
Bacterial spores	Chlorhexidine, QACs, phenolics	Spore coat(s) and cortex present a barrier to entry of antiseptics and disinfectants
Gram-positive bacteria	Chlorhexidine	Glycocalyx/mucoexopolysaccaride may be associated with reduced diffusion of antiseptic
Inactivation (chromosomally mediated)	Chlorohexidine	Breakdown of chlorhexidine molecule may be responsible for resistance

is thus a natural, chromosomally controlled property of a bacterial cell that enables it to circumvent the action of an antiseptic or disinfectant. Gram-negative bacteria tend to be more resistant than gram-positive organisms, such as staphylococci (Table 6).

Intrinsic resistance of bacterial spores. Bacterial spores of the genera *Bacillus* and *Clostridium* have been widely studied and are invariably the most resistant of all types of bacteria to antiseptics and disinfectants (43, 46, 150, 414, 418, 420, 422, 423, 457). Although *Bacillus* species are generally not pathogenic, their spores are widely used as indicators of efficient sterilization. *Clostridium* species are significant pathogens; for example, *C. difficile* is the most common cause of hospital-acquired diarrhea (478). Many biocides are bactericidal or bacteristatic at low concentrations for nonsporulating bacteria, including the vegetative cells of *Bacillus* and *Clostridium* species, but high concentrations may be necessary to achieve a sporicidal effect (e.g., for glutaraldehyde and CRAs). By contrast, even high concentrations of alcohol, phenolics, QACs, and chlorhexidine lack a sporicidal effect, although this may be achieved when these compounds are used at elevated temperatures (475).

A typical spore has a complex structure (29, 151). In brief, the germ cell (protoplast or core) and germ cell wall are surrounded by the cortex, outside which are the inner and outer spore coats. A thin exosporium may be present in the spores of some species but may surround just one spore coat. RNA, DNA, and DPA, as well as most of the calcium, potassium, manganese, and phosphorus, are present in the spore protoplast. Also present are large amounts of low-molecular-weight basic proteins (small acid-soluble spore proteins [SASPs]), which are rapidly degraded during germination. The cortex consists largely of peptidoglycan, including a spore-specific muramic lactam. The spore coats comprise a major portion of the spore. These structures consist largely of protein, with an alkali-soluble fraction made up of acidic polypeptides being found in the inner coat and an alkali-resistant fraction associated with the presence of disulfide-rich bonds being found in the outer coat. These aspects, especially the roles of the coat(s) and cortex, are all relevant to the mechanism(s) of resistance presented by bacterial spores to antiseptics and disinfectants.

Several techniques are available for studying mechanisms of spore resistance (428). They include removing the spore coat and cortex by using a "step-down" technique to achieve a highly synchronous sporulation (so that cellular changes can be accurately monitored), employing spore mutants that do not sporulate beyond genetically determined stages in sporulation, adding an antiseptic or disinfectant at the commencement of sporulation and determining how far the process can proceed, and examining the role of SASPs. Such procedures have helped provide a considerable amount of useful information. Sporulation itself is a process in which a vegetative cell develops into a spore and involves seven stages (designated 0 to VII). During this process, the vegetative cell (stage 0) undergoes a series of morphological changes that culminate in the release of a mature spore (stage VII). Stages IV (cortex development) to VII are the most important in the development of resistance to biocides.

Resistance to antiseptics and disinfectants develops during sporulation and may be an early, intermediate, or (very) late event (103, 375, 378, 429, 474). Useful markers for monitoring the development of resistance are toluene (resistance to which is an early event), heat (intermediate), and lysozyme (late) (236, 237). Studies with a wild-type *B. subtilis* strain, 168, and its Spo^- mutants have helped determine the stages at which resistance develops (262, 375, 474). From these studies (Fig. 2), the order of development of resistance was toluene (marker), formaldehyde, sodium lauryl sulfate, phenol, and phenylmercuric nitrate; *m*-cresol, chlorocresol, chlorhexidine gluconate, cetylpyridinium chloride, and mercuric chloride; and moist heat (marker), sodium dichloroisocyanurate, sodium hypochlorite, lysozyme (marker), and glutaraldehyde. The association of the onset of resistance to a particular antiseptic or disinfectant with a particular stage(s) in spore development is thereby demonstrated.

Spore coat-less forms, produced by treatment of spores un-

TABLE 6. MIC of some antiseptics and disinfectants against gram-positive and gram-negative bacteria[a]

Chemical agent	MIC (μg/ml) for:		
	S. aureus[b]	*E. coli*	*P. aeruginosa*
Benzalkonium chloride	0.5	50	250
Benzethonium chloride	0.5	32	250
Cetrimide	4	16	64–128
Chlorhexidine	0.5–1	1	5–60
Hexachlorophene	0.5	12.5	250
Phenol	2,000	2,000	2,000
o-Phenylphenol	100	500	1,000
Propamine isethionate	2	64	256
Dibromopropamidine isethionate	1	4	32
Triclosan	0.1	5	>300

[a] Based on references 226 and 440.
[b] MICs of cationic agents for some MRSA strains may be higher (see Table 10).

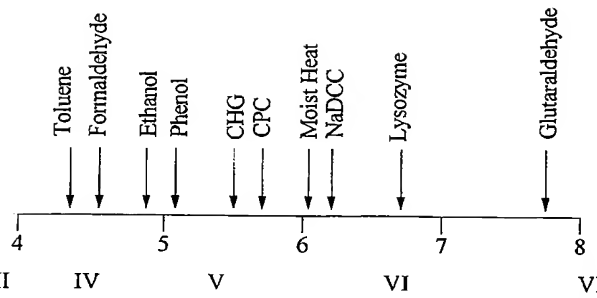


FIG. 2. Development of resistance of *Bacillus subtilis* during sporulation. Roman numerals indicate the sporulation stage from III (engulfment of the forespore) to VII (release of the mature spore). Arabic numbers indicate the time (hours) following the onset of sporulation and the approximate times at which resistance develops against biocides (262). CHG, chlorhexidine; CPC, cetylpyridinium chloride; NaDCC, sodium dichloroisocyanurate.

der alkaline conditions with urea plus dithiothreitol plus sodium lauryl sulfate (UDS), have also been of value in estimating the role of the coats in limiting the access of antiseptics and disinfectants to their target sites. However, Bloomfield and Arthur (44, 45) and Bloomfield (43) showed that this treatment also removes a certain amount of cortex and that the amount of cortex remaining can be further reduced by the subsequent use of lysozyme. These findings demonstrate that the spore coats have an undoubted role in conferring resistance but that the cortex also is an important barrier since (UDS plus lysozyme)-treated spores are much more sensitive to chlorine- and iodine-releasing agents than are UDS-exposed spores.

The initial development and maturity of the cortex are implicated in the development of resistance to phenolics. Likewise, it is now clear that cortex development is at least partially responsible for resistance to chlorhexidine and QACs; this resistance is enhanced in developing spores by the initiation of spore coat synthesis (262). The effect of various concentrations of chlorhexidine, sublethal to vegetative bacteria, on the development of spores of *B. subtilis* 168 MB_2 were investigated by Knott and Russell (261). They found that chlorhexidine affected spore development; as concentrations of the biguanide increased, spore index values (the percentage of cells forming spores) decreased and sensitivity to both heat and toluene increased. By contrast, the control (untreated) culture was highly resistant to both of these agents and had a high spore index value, indicative of high levels of mature spores. The slightly increased resistance to toluene compared to resistance to heat was not surprising, since cells must reach stages V to VI (synthesis of spore coats and maturation) to attain heat resistance but only stage III (forespore engulfment) to attain toluene resistance (Fig. 2); in other words, if sporulation is inhibited by chlorhexidine, more cells are likely to reach stage III than the later stages. While less definitive than the earlier approaches, these procedures provide further evidence of the involvement of the cortex and coats in chlorhexidine resistance.

Development of resistance during sporulation to formaldehyde was an early event but depended to some extent on the concentration (1 to 5% [vol/vol]) of formaldehyde used. This appears to be at odds with the extremely late development of resistance to the dialdehyde, glutaraldehyde. Since glutaraldehyde and the monoaldehyde, formaldehyde, contain an aldehyde group(s) and are alkylating agents, it would be plausible to assume that they would have a similar mode of sporicidal action, even though the dialdehyde is a more powerful alkylating agent. If this were true, it could also be assumed that spores would exhibit the same resistance mechanisms for these disinfectants. In aqueous solution, formaldehyde forms a glycol in equilibrium (512, 524); thus, formaldehyde could well be acting poorly as an alcohol-type disinfectant rather than as an aldehyde (327). Alkaline glutaraldehyde does not readily form glycols in aqueous solution (178). Resistance to formaldehyde may be linked to cortex maturation, and resistance to glutaraldehyde may be linked to coat formation (262).

Setlow and his coworkers (472) demonstrated that α/β-type SASPs coat the DNA in wild-type spores of *B. subtilis*, thereby protecting it from attack by enzymes and antimicrobial agents. Spores ($\alpha^-\beta^-$) lacking these α/β-type SASPs are significantly more sensitive to hydrogen peroxide (471) and hypochlorite (456). Thus, SASPs contribute to spore resistance to peroxide and hypochlorite but may not be the only factors involved, since the coats and cortex also play a role (428).

Two other aspects of spores should be considered: the revival of injured spores and the effects of antiseptics and disinfectants on germinating and outgrowing spores. Although neither aspect is truly a resistance mechanism, each can provide useful information about the site and mechanism of action of sporicidal agents and about the associated spore resistance mechanisms and might be of clinical importance.

The revival of disinfectant-treated spores has not been extensively studied. Spicher and Peters (483, 484) demonstrated that formaldehyde-exposed spores of *B. subtilis* could be revived after a subsequent heat shock process. A more recent finding with *B. stearothermophilus* casts further doubt on the efficacy of low-temperature steam with formaldehyde as a sterilizing procedure (541). The revival of spores exposed to glutaraldehyde, formaldehyde, chlorine, and iodine was examined by Russell and his colleagues (103, 376, 377, 424, 532–537). A small proportion of glutaraldehyde-treated spores of various *Bacillus* species were revived when the spores were treated with alkali after neutralization of glutaraldehyde with glycine (103, 379, 380). Experiments designed to distinguish between germination and outgrowth in the revival process have demonstrated that sodium hydroxide-induced revival increases the potential for germination. Based on other findings, the germination process is also implicated in the revival of spores exposed to other disinfectants.

Intrinsic resistance of mycobacteria. Mycobacteria are well known to possess a resistance to antiseptics and disinfectants that is roughly intermediate between those of other nonsporulating bacteria and bacterial spores (Fig. 1) (177, 345, 419). There is no evidence that enzymatic degradation of harmful molecules takes place. The most likely mechanism for the high resistance of mycobacteria is associated with their complex cell walls that provide an effective barrier to the entry of these agents. To date, plasmid- or transposon-mediated resistance to biocides has not been demonstrated in mycobacteria.

The mycobacterial cell wall is a highly hydrophobic structure with a mycoylarabinogalactan-peptidoglycan skeleton (27, 105, 106, 322, 389, 390, 461, 530). The peptidoglycan is covalently linked to the polysaccharide copolymer (arabinogalactan) made up of arabinose and galactose esterified to mycolic acids. Also present are complex lipids, lipopolysaccharides (LPSs), and proteins, including porin channels through which hydrophilic molecules can diffuse into the cell (232, 356). Similar cell wall structures exist in all the mycobacterial species examined to date (228). The cell wall composition of a particular species may be influenced by its environmental niche (27). Pathogenic bacteria such as *Mycobacterium tuberculosis* exist in a relatively nutrient-rich environment, whereas saprophytic mycobacteria living in soil or water are exposed to natural antibiotics and tend to be more intrinsically resistant to these drugs.

Antiseptics or disinfectants that exhibit mycobacterial activity are phenol, PAA, hydrogen peroxide, alcohol, and glutaraldehyde (16, 17, 99, 419, 425, 455). By contrast, other well-known bactericidal agents, such as chlorhexidine and QACs, are mycobacteristatic even when used at high concentrations (51, 52, 419, 425, 455). However, the activity of these can be substantially increased by formulation effects. Thus, a number of QAC-based products claim to have mycobacterial activity. For example, a newer formulation (Sactimed-I-Sinald) containing a mixture of alkyl polyguanides and alkyl QACs is claimed to be mycobactericidal (211, 353). However, there is some doubt whether the antibacterial agents had been properly quenched or neutralized to prevent carryover of inhibitory concentrations into recovery media.

Many years ago, it was proposed (T. H. Shen, cited in reference 99) that the resistance of mycobacteria to QACs was related to the lipid content of the cell wall. In support of this contention, *Mycobacterium phlei*, which has a low total cell lipid content, was more sensitive than *M. tuberculosis*, which has a higher lipid content. It was also noted that the resistance of various species of mycobacteria was related to the content of waxy material in the wall. It is now known that because of the highly hydrophobic nature of the cell wall, hydrophilic biocides are generally unable to penetrate the mycobacterial cell wall in sufficiently high concentrations to produce a lethal effect. However, low concentrations of antiseptics and disinfectants such as chlorhexidine must presumably traverse this permeability barrier, because the MICs are of the same order as those concentrations inhibiting the growth of nonmycobacterial strains such as *S. aureus*, although *M. avium-intracellulare* may be particularly resistant (51, 52). The component(s) of the mycobacterial cell wall responsible for the high biocide resistance are currently unknown, although some information is available. Inhibitors of cell wall synthesis increase the susceptibility of *M. avium* to drugs (391); inhibition of myocide C, arabinogalactan, and mycolic acid biosynthesis enhances drug susceptibility. Treatment of this organism with *m*-fluoro-DL-phenylalanine (*m*-FL-phe), which inhibits mycocide C synthesis, produces significant alterations in the outer cell wall layers (106). Ethambutol, an inhibitor of arabinogalactan (391, 501) and phospholipid (461, 462) synthesis, also disorganizes these layers. In addition, ethambutol induces the formation of ghosts without the dissolution of peptidoglycan (391). Methyl-4-(2-octadecylcyclopropen-1-yl) butanoate (MOCB) is a structural analogue of a key precursor in mycolic acid synthesis. Thus, the effects of MOCB on mycolic acid synthesis and *m*-FL-phe and ethambutol on outer wall biosynthetic processes leading to changes in cell wall architecture appear to be responsible for increasing the intracellular concentration of chemotherapeutic drugs. These findings support the concept of the cell wall acting as a permeability barrier to these drugs (425). Fewer studies have been made of the mechanisms involved in the resistance of mycobacteria to antiseptics and disinfectants. However, the activity of chlorhexidine and of a QAC, cetylpyridinium chloride, against *M. avium* and *M. tuberculosis* can be potentiated in the presence of ethambutol (52). From these data, it may be inferred that arabinogalactan is one cell wall component that acts as a permeability barrier to chlorhexidine and QACs. It is not possible, at present, to comment on other components, since these have yet to be investigated. It would be useful to have information about the uptake into the cells of these antiseptic agents in the presence and absence of different cell wall synthesis inhibitors.

One species of mycobacteria currently causing concern is *M. chelonae*, since these organisms are sometimes isolated from endoscope washes and dialysis water. One such strain was not killed even after a 60-min exposure to alkaline glutaraldehyde; in contrast, a reference strain showed a 5-log-unit reduction after a contact time of 10 min (519). This glutaraldehyde-resistant *M. chelonae* strain demonstrated an increased tolerance to PAA but not to NaDCC or to a phenolic. Other workers have also observed an above-average resistance of *M. chelonae* to glutaraldehyde and formaldehyde (72) but not to PAA (187, 294). The reasons for this high glutaraldehyde resistance are unknown. However, *M. chelonae* is known to adhere strongly to smooth surfaces, which may render cells within a biofilm less susceptible to disinfectants. There is no evidence to date that uptake of glutaraldehyde by *M. chelonae* is reduced.

Intrinsic resistance of other gram-positive bacteria. The cell wall of staphylococci is composed essentially of peptidoglycan and teichoic acid. Neither of these appears to act as an effective barrier to the entry of antiseptics and disinfectants. Since high-molecular-weight substances can readily traverse the cell wall of staphylococci and vegetative *Bacillus* spp., this may explain the sensitivity of these organisms to many antibacterial agents including QACs and chlorhexidine (411, 417, 422, 428, 451).

However, the plasticity of the bacterial cell envelope is a well-known phenomenon (381). Growth rate and any growth-limiting nutrient will affect the physiological state of the cells. Under such circumstances, the thickness and degree of cross-linking of peptidoglycan are likely to be modified and hence the cellular sensitivity to antiseptics and disinfectants will be altered. For example, Gilbert and Brown (171) demonstrated that the sensitivity of *Bacillus megaterium* cells to chlorhexidine and 2-phenoxyethanol is altered when changes in growth rate and nutrient limitation are made with chemostat-grown cells. However, lysozyme-induced protoplasts of these cells remained sensitive to, and were lysed by, these membrane-active agents. Therefore, the cell wall in whole cells is responsible for their modified response.

In nature, *S. aureus* may exist as mucoid strains, with the cells surrounded by a slime layer. Nonmucoid strains are killed more rapidly than mucoid strains by chloroxylenol, cetrimide, and chlorhexidine, but there is little difference in killing by phenols or chlorinated phenols (263); removal of slime by washing rendered the cells sensitive. Therefore, the slime plays a protective role, either as a physical barrier to disinfectant penetration or as a loose layer interacting with or absorbing the biocide molecules.

There is no evidence to date that vancomycin-resistant enterococci or enterococci with high-level resistance to aminoglycoside antibiotics are more resistant to disinfectants than are antibiotic-sensitive enterococcal strains (9, 11, 48, 319). However, enterococci are generally less sensitive to biocides than are staphylococci, and differences in inhibitory and bactericidal concentrations have also been found among enterococcal species (257).

Intrinsic resistance of gram-negative bacteria. Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than are nonsporulating, nonmycobacterial gram-positive bacteria (Fig. 2) (428, 440, 441). Examples of MICs against gram-positive and -negative organisms are provided in Table 6. Based on these data, there is a marked difference in the sensitivity of *S. aureus* and *E. coli* to QACs (benzalkonium, benzethonium, and cetrimide), hexachlorophene, diamidines, and triclosan but little difference in chlorhexidine susceptibility. *P. aeruginosa* is considerably more resistant to most of these agents, including chlorhexidine, and (not shown) *Proteus* spp. possess an above-average resistance to cationic agents such as chlorhexidine and QACs (311, 440).

The outer membrane of gram-negative bacteria acts as a barrier that limits the entry of many chemically unrelated types

TABLE 7. Possible transport of some antiseptics and disinfectants into gram-negative bacteria[a]

Antiseptic/disinfectant	Passage across OM[b]	Passage across IM[b]
Chlorhexidine	Self-promoted uptake(?)	IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs
QACs	Self-promoted uptake(?); also, OM might present a barrier	IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs
Phenolics	Hydrophobic pathway (activity increases as hydrophobicity of phenolic increases)	IM is a major target site, but high phenolic concentrations coagulate cytoplasmic constituents

[a] Data from references 197, 433 to 435, 438, and 439.
[b] OM, outer membrane; IM, inner membrane.

of antibacterial agents (18, 169, 196, 197, 355, 366, 440, 516, 517). This conclusion is based on the relative sensitivities of staphylococci and gram-negative bacteria and also on studies with outer membrane mutants of *E. coli*, *S. typhimurium*, and *P. aeruginosa* (134, 135, 433–435, 438). Smooth, wild-type bacteria have a hydrophobic cell surface; by contrast, because of the phospholipid patches on the cell surface, deep rough (heptose-less) mutants are hydrophobic. These mutants tend to be hypersensitive to hydrophobic antibiotics and disinfectants. Low-molecular-weight (M_r <ca. 600) hydrophilic molecules readily pass via the porins into gram-negative cells, but hydrophobic molecules diffuse across the outer membrane bilayer (Table 7). In wild-type gram-negative bacteria, intact LPS molecules prevent ready access of hydrophobic molecules to phospholipid and thence to the cell interior. In deep rough strains, which lack the O-specific side chain and most of the core polysaccharide, the phospholipid patches at the cell surface have their head groups oriented toward the exterior.

In addition to these hydrophilic and hydrophobic entry pathways, a third pathway has been proposed for cationic agents such as QACs, biguanidies, and diamidines. It is claimed that these damage the outer membrane, thereby promoting their own uptake (197). Polycations disorganize the outer membrane of *E. coli* (520). It must be added, however, that the QACs and diamidines are considerably less active against wild-type strains than against deep rough strains whereas chlorhexidine has the same order of activity (MIC increase about 2 to 3 fold) against both types of *E. coli* strains (434, 435, 439). However, *S. typhimurium* mutants are more sensitive to chlorhexidine than are wild-type strains (433).

Gram-negative bacteria that show a high level of resistance to many antiseptics and disinfectants include *P. aeruginosa*, *Burkholderia cepacia*, *Proteus* spp., and *Providencia stuartii* (428, 440). The outer membrane of *P. aeruginosa* is responsible for its high resistance; in comparison with other organisms, there are differences in LPS composition and in the cation content of the outer membrane (54). The high Mg^{2+} content aids in producing strong LPS-LPS links; furthermore, because of their small size, the porins may not permit general diffusion through them. *B. cepacia* is often considerably more resistant in the hospital environment than in artificial culture media (360); the high content of phosphate-linked arabinose in its LPS decreases the affinity of the outer membrane for polymyxin antibiotics and other cationic and polycationic molecules (97, 516). *Pseudomonas stutzeri*, by contrast, is highly sensitive to many antibiotics and disinfectants (449), which implies that such agents have little difficulty in crossing the outer layers of the cells of this organism.

Members of the genus *Proteus* are invariably insensitive to chlorhexidine (311). Some strains that are highly resistant to chlorhexidine, QACs, EDTA, and diamidines have been isolated from clinical sources. The presence of a less acidic type of outer membrane LPS could be a contributing factor to this intrinsic resistance (97, 516).

A particularly troublesome member of the genus *Providencia* is *P. stuartii*. Like *Proteus* spp., *P. stuartii* strains have been isolated from urinary tract infections in paraplegic patients and are resistant to different types of antiseptics and disinfectants including chlorhexidine and QACs (492, 496). Strains of *P. stuartii* that showed low-, intermediate-, and high-level resistance to chlorhexidine formed the basis of a series of studies of the resistance mechanism(s) (86, 422, 428). Gross differences in the composition of the outer layers of these strains were not detected, and it was concluded that (i) subtle changes in the structural arrangement of the cell envelopes of these strains was associated with this resistance and (ii) the inner membrane was not implicated (230).

Few authors have considered peptidoglycan in gram-negative bacteria as being a potential barrier to the entry of inhibitory substances. The peptidoglycan content of these organisms is much lower than in staphylococci, which are inherently more sensitive to many antiseptics and disinfectants. Nevertheless, there have been instances (discussed in reference 422) where gram-negative organisms grown in subinhibitory concentrations of a penicillin have deficient permeability barriers. Furthermore, it has been known for many years (215, 409, 411) that penicillin-induced spheroplasts and lysozyme-EDTA-Tris "protoplasts" of gram-negative bacteria are rapidly lysed by membrane-active agents such as chlorhexidine. It is conceivable that the stretched nature of both the outer and inner membranes in β-lactam-treated organisms could contribute to this increased susceptibility.

The possibility exists that the cytoplasmic (inner) membrane provides one mechanism of intrinsic resistance. This membrane is composed of lipoprotein and would be expected to prevent passive diffusion of hydrophilic molecules. It is also known that changes in membrane composition affect sensitivity to ethanol (159). Lannigan and Bryan (275) proposed that decreased susceptibility of *Serratia marcescens* to chlorhexidine was linked to the inner membrane, but Ismaeel et al. (230) could find no such role with chlorhexidine-resistant *P. stuartii*. At present, there is little evidence to implicate the inner membrane in biocide resistance. In addition, chlorhexidine degradation was reported for *S. marcescens*, *P. aeruginosa*, and *Achromobacter/Alcaligenes xylosoxidans* (358).

Physiological (phenotypic) adaption as an intrinsic mechanism. The association of microorganisms with solid surfaces leads to the generation of a biofilm, defined as a consortium of organisms organized within an extensive exopolysaccharide exopolymer (93, 94). Biofilms can consist of monocultures, of several diverse species, or of mixed phenotypes of a given species (57, 73, 381). Some excellent publications that deal with the nature, formation, and content of biofilms are available (125, 178, 276, 538). Biofilms are important for several reasons,

TABLE 8. Biofilms and microbial response to antimicrobial agents

Mechanism of resistance associated with biofilms	Comment
Exclusion or reduced access of antiseptic or disinfectant to underlying cell	Depends on (i) nature of antiseptic/disinfectant, (ii) binding capacity of glycocalyx toward antiseptic or disinfectant, and (iii) rate of growth of microcolony relative to diffusion rate of chemical inhibitor
Modulation of microenvironment	Associated with (i) nutrient limitation and (ii) growth rate
Increased production of degradative enzymes by attached cells	Mechanism unclear at present
Plasmid transfer between cells in biofilm?	Associated with enhanced tolerance to antiseptics and disinfectants?

notably biocorrosion, reduced water quality, and foci for contamination of hygienic products (10, 12–14). Colonization also occurs on implanted biomaterials and medical devices, resulting in increased infection rates and possible recurrence of infection (125).

Bacteria in different parts of a biofilm experience different nutrient environments, and their physiological properties are affected (57). Within the depths of a biofilm, for example, nutrient limitation is likely to reduce growth rates, which can affect susceptibility to antimicrobial agents (98, 142, 171, 172). Thus, the phenotypes of sessile organisms within biofilms differ considerably from the planktonic cells found in laboratory cultures (73). Slow-growing bacteria are particularly insusceptible, a point reiterated recently in another context (126).

Several reasons can account for the reduced sensitivity of bacteria within a biofilm (Table 8). There may be (i) reduced access of a disinfectant (or antibiotic) to the cells within the biofilm, (ii) chemical interaction between the disinfectant and the biofilm itself, (iii) modulation of the microenvironment, (iv) production of degradative enzymes (and neutralizing chemicals), or (v) genetic exchange between cells in a biofilm. However, bacteria removed from a biofilm and recultured in culture media are generally no more resistant than the "ordinary" planktonic cells of that species (57).

Several instances are known of the contamination of antiseptic or disinfectant solutions by bacteria. For example, Marrie and Costerton (310) described the prolonged survival of *S. marcescens* in 2% chlorhexidine solutions, which was attributed to the embedding of these organisms in a thick matrix that adhered to the walls of a storage containers. Similar conclusions were reached by Hugo et al. (225) concerning the survival of *B. cepacia* in chlorhexidine and by Anderson et al. (10, 12–14) concerning the contamination of iodophor antiseptics with *Pseudomonas*. In the studies by Anderson et al., *Pseudomonas* biofilms were found on the interior surfaces of polyvinyl chloride pipes used during the manufacture of providone-iodine antiseptics. It is to be wondered whether a similar reason could be put forward for the contamination by *S. marcescens* of a benzalkonium chloride solution implicated in meningitis (468). Recently, a novel strategy was described (540) for controlling biofilms through generation of hydrogen peroxide at the biofilm-surface interface rather than simply applying a disinfectant extrinsically. In this procedure, the colonized surface incorporated a catalyst that generated the active compound from a treatment agent.

Gram-negative pathogens can grow as biofilms in the catheterized bladder and are able to survive concentrations of chlorhexidine that are effective against organisms in noncatheterized individuals (493, 494). Interestingly, the permeability agent EDTA has only a temporary potentiating effect in the catheterized bladder, with bacterial growth subsequently recurring (495). *B. cepacia* freshly isolated from the hospital environment is often considerably more resistant to chlorhexidine than when grown in artificial culture media, and a glycocalyx may be associated with intrinsic resistance to the bisbiguanide (360). *Legionella pneumophila* is often found in hospital water distribution systems and cooling towers. Chlorination in combination with continuous heating (60°C) of incoming water is usually the most important disinfection measure; however, because of biofilm production, contaminating organisms may be less susceptible to this treatment (140). Increased resistance to chlorine has been reported for *Vibrio cholerae*, which expresses an amorphous exopolysaccharide causing cell aggregation ("rugose" morphology [336]) without any loss in pathogenicity.

One can reach certain conclusions about biofilms. The interaction of bacteria with surfaces is usually reversible and eventually irreversible. Irreversible adhesion is initiated by the binding of bacteria to the surface through exopolysaccharide glycocalyx polymers. Sister cells then arise by cell division and are bound within the glycocalyx matrix. The development of adherent microcolonies is thereby initiated, so that eventually a continuous biofilm is produced on the colonized surface. Bacteria within these biofilms reside in specific microenvironments that differ from those of cells grown under normal laboratory conditions and thus show variations in their response to antiseptics and disinfectants.

Recent nosocomial outbreaks due to *M. chelonae* (discussed under "Intrinsic resistance of mycobacteria"), *M. tuberculosis* (4, 323) and HCV (53) underscore the importance of pseudobiofilm formation in flexible fiberoptic scope contamination. These outbreaks were associated with inadequate cleaning of scopes, which compromised subsequent sterilization with glutaraldehyde. While these organisms do not form a true biofilm, the cross-linking action of glutaraldehyde can cause a buildup of insoluble residues and associated microorganisms on scopes and in automated reprocessors.

Biofilms provide the most important example of how physiological (phenotypic) adaptation can play a role in conferring intrinsic resistance (57). Other examples are also known. For example, fattened cells of *S. aureus* produced by repeated subculturing in glycerol-containing media are more resistant to alkyl phenols and benzylpenicillin than are wild-type strains (220). Subculture of these cells in routine culture media resulted in reversion to sensitivity (218). Planktonic cultures grown under conditions of nutrient limitation or reduced growth rates have cells with altered sensitivity to disinfectants, probably as a consequence of modifications in their outer membranes (56, 59, 98). In addition, many aerobic microorganisms have developed intrinsic defense systems that confer tolerance to peroxide stress (in particular H_2O_2) in vivo. The so-called oxidative-stress or SOS response has been well studied in *E. coli* and *Salmonella* and includes the production of neutralizing enzymes to prevent cellular damage (including peroxidases, catalases, glutathione reductase) and to repair DNA lesions (e.g., exonuclease III) (112, 114, 497). In both organisms, increased tolerance can be obtained by pretreatment with a subinhibitory dose of hydrogen peroxide (113, 539). Pretreatment induces a series of proteins, many of which are under the positive control of a sensor/regulator protein (OxyR), including catalase and glutathione reductase (497)

TABLE 9. Possible mechanisms of plasmid-encoded resistance to antiseptics and disinfectants

Chemical agent	Examples	Mechanisms
Antiseptics or disinfectants	Chlorhexidine salts	(i) Inactivation: not yet found to be plasmid mediated; chromosomally mediated inactivation; (ii) efflux: some *S. aureus*, some *S. epidermidis*; (iii) Decreased uptake(?)
	QACs	(i) Efflux: some *S. aureus*, some *S. epidermidis*; (ii) Decreased uptake(?)
	Silver compounds	Decreased uptake; no inactivation (cf. mercury compounds)
	Formaldehyde	(i) Inactivation by formaldehyde dehydrogenase; (ii) Cell surface alterations (outer membrane proteins)
	Acridines[a]	Efflux: some *S. aureus*, some *S. epidermidis*
	Diamidines	Efflux: some *S. aureus*, some *S. epidermidis*
	Crystal violet[a]	Efflux: some *S. aureus*, some *S. epidermidis*
Other biocides	Mercurials[b]	Inactivation (reductases, lyases)
	Ethidium bromide	Efflux: some *S. aureus*, some *S. epidermidis*

[a] Now rarely used for antiseptic or disinfectant purposes.
[b] Organomercurials are still used as preservatives.

and further nonessential proteins that accumulate to protect the cell (338). Cross-resistance to heat, ethanol, and hypochlorous acid has also been reported (81, 128, 335). The oxidative stress response in gram-positive bacteria is less well studied, but *Bacillus* tolerance to H_2O_2 has been described to vary during the growth phase (127) and in mutant strains (67, 200). Similar inducible defense mechanisms were described for *Campylobacter jejuni* (185), *Deinococcus* (528), and *Haemophilus influenzae* (36). However, the level of increased tolerance to H_2O_2 during the oxidative stress response may not afford significant protection to concentrations used in antiseptics and disinfectants (generally >3%). For example, *B. subtilis* mutants have been described to be more resistant at ~0.5% H_2O_2 than are wild-type strains at ~0.34% H_2O_2 (200).

Acquired Bacterial Resistance Mechanisms

As with antibiotics and other chemotherapeutic drugs, acquired resistance to antiseptics and disinfectants can arise by either mutation or the acquisition of genetic material in the form of plasmids or transposons. It is important to note that "resistance" as a term can often be used loosely and in many cases must be interpreted with some prudence. This is particularly true with MIC analysis. Unlike antibiotics, "resistance," or an increase in the MIC of a biocide, does not necessarily correlate with therapeutic failure. An increase in an antibiotic MIC can may have significant consequences, often indicating that the target organism is unaffected by its antimicrobial action. Increased biocide MICs due to acquired mechanisms have also been reported and in some case misinterpreted as indicating resistance. It is important that issues including the pleiotropic action of most biocides, bactericidal activity, concentrations used in products, direct product application, formulation effects, etc., be considered in evaluating the clinical implications of these reports.

Plasmids and bacterial resistance to antiseptics and disinfectants. Chopra (82, 83) examined the role of plasmids in encoding resistance (or increased tolerance) to antiseptics and disinfectants; this topic was considered further by Russell (413). It was concluded that apart from certain specific examples such as silver, other metals, and organomercurials, plasmids were not normally responsible for the elevated levels of antiseptic or disinfectant resistance associated with certain species or strains. Since then, however, there have been numerous reports linking the presence of plasmids in bacteria with increased tolerance to chlorhexidine, QACs, and triclosan, as well as to diamidines, acridines and ethidium bromide, and the topic was reconsidered (83, 423, 427) (Table 9).

Plasmid-encoded resistance to antiseptics and disinfectants had at one time been most extensively investigated with mercurials (both inorganic and organic), silver compounds, and other cations and anions. Mercurials are no longer used as disinfectants, but phenylmercuric salts and thiomersal are still used as preservatives in some types of pharmaceutical products (226). Resistance to mercury is plasmid borne, inducible, and may be transferred by conjugation or transduction. Inorganic mercury (Hg^{2+}) and organomercury resistance is a common property of clinical isolates of *S. aureus* containing penicillinase plasmids (110). Plasmids conferring resistance to mercurials are either narrow spectrum, specifying resistance to Hg^{2+} and to some organomercurials, or broad-spectrum, with resistance to the above compounds and to additional organomercurials (331). Silver salts are still used as topical antimicrobial agents (50, 443). Plasmid-encoded resistance to silver has been found in *Pseudomonas stutzeri* (192), members of the *Enterobacteriaceae* (479, 480, 511), and *Citrobacter* spp. (511). The mechanism of resistance has yet to be elucidated fully but may be associated with silver accumulation (152, 511).

(i) Plasmid-mediated antiseptic and disinfectant resistance in gram-negative bacteria. Occasional reports have examined the possible role of plasmids in the resistance of gram-negative bacteria to antiseptics and disinfectants. Sutton and Jacoby (498) observed that plasmid RP1 did not significantly alter the resistance of *P. aeruginosa* to QACs, chlorhexidine, iodine, or chlorinated phenols, although increased resistance to hexachlorophene was observed. This compound has a much greater effect on gram-positive than gram-negative bacteria, so that it is difficult to assess the significance of this finding. Transformation of this plasmid (which encodes resistance to carbenicillin, tetracycline, and neomycin and kanamycin) into *E. coli* or *P. aeruginosa* did not increase the sensitivity of these organisms to a range of antiseptics (5).

Strains of *Providencia stuartii* may be highly tolerant to Hg^{2+}, cationic disinfectants (such as chlorhexidine and QACs), and antibiotics (496). No evidence has been presented to show that there is a plasmid-linked association between antibiotic resistance and disinfectant resistance in these organisms, pseudomonads, or *Proteus* spp. (492). High levels of disinfectant resistance have been reported in other hospital isolates (195), although no clear-cut role for plasmid-specified resistance has emerged (102, 250, 348, 373, 518). High levels of tolerance to chlorhexidine and QACs (311) may be intrinsic or may have resulted from mutation. It has been proposed (492, 496) that the extensive usage of these cationic agents could be responsible for the selection of antiseptic-disinfectant-, and antibiotic-resistant strains; however, there is little evidence to support this conclusion. All of these studies demonstrated that it was difficult to transfer chlorhexidine or QAC resistance under nor-

TABLE 10. *qac* genes and susceptibility of *S. aureus* strains to some antiseptics and disinfectants

qac gene[a]	MIC ratios[b] of[c]:							
	Proflavine	CHG	Pt	Pi	CTAB	BZK	CPC	DC
qacA	>16	2.5	>16	>16	4	>3	>4	2
qacB	8	1	>4	2	2	>3	>2	2
qacC	1	1	ca. 1	1	6	>3	>4	1
qacD	1	1	ca. 1	1	6	>3	>4	1
MIC (μg/ml) for sensitive strain	40	0.8	<50	50[d]	1	<2	<1	4

[a] *qac* genes are otherwise known as nucleic acid binding (NAB) compound resistance genes (88).
[b] Calculated from the data in reference 289. Ratios are MICs for strains of *S. aureus* carrying various *qac* genes divided by the MIC for a strain carrying no gene (the actual MIC for the test strain is shown in the bottom row).
[c] CHG, chlorhexidine diacetate; Pt, pentamidine isethionate; Pi, propamidime isethionate; CTAB, cetyltrimethylammonium bromide; BZK, benzalkonium chloride; CPC, cetylpyridinium chloride; DC, dequalinium chloride.
[d] The MIC of propamidine isethionate for the sensitive *S. aureus* is considerably higher than the normal quoted value (ca. 2 μg/ml [Table 6]).

mal conditions and that plasmid-mediated resistance to these chemicals in gram-negative bacteria was an unlikely event. By contrast, plasmid R124 alters the OmpF outer membrane protein in *E. coli*, and cells containing this plasmid are more resistant to a QAC (cetrimide) and to other agents (406).

Bacterial resistance mechanisms to formaldehyde and industrial biocides may be plasmid encoded (71, 193). Alterations in the cell surface (outer membrane proteins [19, 246]) and formaldehyde dehydrogenase (247, 269) are considered to be responsible (202). In addition, the so-called TOM plasmid encodes enzymes for toluene and phenol degradation in *B. cepacia* (476).

(ii) Plasmid-mediated antiseptic and disinfectant resistance in staphylococci. Methicillin-resistant *S. aureus* (MRSA) strains are a major cause of sepsis in hospitals throughout the world, although not all strains have increased virulence. Many can be referred to as "epidemic" MRSA because of the ease with which they can spread (91, 295, 317). Patients at particularly high risk are those who are debilitated or immunocompromised or who have open sores.

It has been known for several years that some antiseptics and disinfectants are, on the basis of MICs, somewhat less inhibitory to *S. aureus* strains that contain a plasmid carrying genes encoding resistance to the aminoglycoside antibiotic gentamicin (Table 10). These biocidal agents include chlorhexidine, diamidines, and QACs, together with ethidium bromide and acridines (8, 238, 289, 368, 423, 427, 463). According to Mycock (346), MRSA strains showed a remarkable increase in tolerance (at least 5,000-fold) to povidone-iodine. However, there was no decrease in susceptibility of antibiotic-resistant strains to phenolics (phenol, cresol, and chlorocresol) or to the preservatives known as parabens (8).

Tennent et al. (505) proposed that increased resistances to cetyltrimethylammonium bromide (CTAB) and to propamidine isethionate were linked and that these cationic agents may be acting as a selective pressure for the retention of plasmids encoding resistance to them. The potential clinical significance of this finding remains to be determined.

Staphylococci are the only bacteria in which the genetic aspects of plasmid-mediated antiseptic and disinfectant resistant mechanisms have been described (466). In *S. aureus*, these mechanisms are encoded by at least three separate multidrug resistance determinants (Tables 10 and 11). Increased antiseptic MICs have been reported to be widespread among MRSA strains and to be specified by two gene families (*qacAB* and *qacCD*) of determinants (188, 280, 281, 288, 289, 363–365, 367, 506). The *qacAB* family of genes (Table 11) encodes proton-dependant export proteins that develop significant homology to other energy-dependent transporters such as the tetracycline transporters found in various strains of tetracycline-resistant bacteria (405). The *qacA* gene is present predominantly on the pSK1 family of multiresistance plasmids but is also likely to be present on the chromosome of clinical *S. aureus* strains as an integrated family plasmid or part thereof. The *qacB* gene is detected on large heavy-metal resistance plasmids. The *qacC* and *qacD* genes encode identical phenotypes and show restriction site homology; the *qacC* gene may have evolved from *qacD* (288).

Interesting studies by Reverdy et al. (395, 396), Dussau et al. (129) and Behr et al. (31) demonstrated a relationship between increased *S. aureus* MICs to the β-lactam oxacillin and four antiseptics (chlorhexidine, benzalkonium chloride, hexamine, and acriflavine). A gene encoding multidrug resistance was not found in susceptible strains but was present in 70% of *S. aureus* strains for which the MICs of all four of these antiseptics were increased and in 45% of the remaining strains resistant to at least one of these antiseptics (31). Genes encoding increased QAC tolerance may be widespread in food-associated staphylococcal species (203). Some 40% of isolates of coagulase-negative staphylococci (CNS) contain both *qacA* and *qacC* genes, with a possible selective advantage in possessing both as opposed to *qacA* only (281). Furthermore, there is growing evidence that *S. aureus* and CNS have a common pool of resistance determinants.

Triclosan is used in surgical scrubs, soaps, and deodorants. It is active against staphylococci and less active against most gram-negative organisms, especially *P. aeruginosa*, probably by virtue of a permeability barrier (428). Low-level transferable resistance to triclosan was reported in MRSA strains (88, 90); however, no further work on these organisms has been described. According to Sasatsu et al. (465), the MICs of triclosan against sensitive and resistant *S. aureus* strains were 100 and

TABLE 11. *qac* genes and resistance to quaternary ammonium compounds and other antiseptics and disinfectants

Multidrug resistance determinant[a]	Gene location	Resistance encoded to
qacA	pSK1 family of multiresistant plasmids, also β-lactamase and heavy-metal resistance families	QACs, chlorhexidine salts, diamidines, acridines, ethidium bromide
qacB	β-Lactamase and heavy-metal resistance plasmids	QACs, acridines, ethidium bromide
qacC[b]	Small plasmids (<3 kb) or large conjugative plasmids	Some QACs, ethidium bromide
qacD[b]	Large (50-kb) conjugative, multiresistance plasmids	Some QACs, ethidium bromide

[a] The *qacK* gene has also been described, but it is likely to be less significant than *qacAB* in terms of antiseptic or disinfectant tolerance.
[b] These genes have identical target sites and show restriction site homology.

>6,400 μg/ml, respectively; these results were disputed because these concentrations are well in excess of the solubility of triclosan (515), which is practically insoluble in water. Sasatsu et al. (464) described a high-level resistant strain of *S. aureus* for which the MICs of chlorhexidine, CTAB, and butylparaben were the same as for a low-level resistant strain. Furthermore, the MIC quoted for methylparaben comfortably exceeds its aqueous solubility. Most of these studies with sensitive and "resistant" strains involved the use of MIC evaluations (for example, Table 6). A few investigations examined the bactericidal effects of antiseptics. Cookson et al. (89) pointed out that curing of resistance plasmids produced a fall in MICs but not a consistent decrease in the lethal activity of chlorhexidine. There is a poor correlation between MIC and the rate of bactericidal action of chlorhexidine (88, 89, 319) and triclosan (90, 319). McDonnell et al. (318, 319) have described methicillin-susceptible *S. aureus* (MSSA) and MRSA strains with increased triclosan MICs (up to 1.6 μg/ml) but showed that the MBCs for these strains were identical; these results were not surprising, considering that biocides (unlike antibiotics) have multiple cellular targets. Irizarry et al. (229) compared the susceptibility of MRSA and MSSA strains to CPC and chlorhexidine by both MIC and bactericidal testing methods. However, the conclusion of this study that MRSA strains were more resistant warrants additional comments. On the basis of rather high actual MICs, MRSA strains were some four times more resistant to chlorhexidine and five times more resistant to a QAC (CPC) than were MSSA strains. At a concentration in broth of 2 μg of CPC/ml, two MRSA strains grew normally with a threefold increase in viable numbers over a 4-h test period whereas an MSSA strain showed a 97% decrease in viability. From this, the authors concluded that it was reasonable to speculate that the residual amounts of antiseptics and disinfectants found in the hospital environment could contribute to the selection and maintenance of multiresistant MRSA strains. Irizarry et al. (229) also concluded that MRSA strains are less susceptible than MSSA strains to both chronic and acute exposures to antiseptics and disinfectants. However, their results with 4 μg of CPC/ml show no such pattern: at this higher concentration, the turbidities (and viability) of the two MRSA and one MSSA strains decreased at very similar rates (if anything, one MRSA strain appeared to be affected to a slightly greater extent that the MSSA strain). Furthermore, the authors stated that chlorhexidine gave similar results to CPC. It is therefore difficult to see how Irizarry et al. arrived at their highly selective conclusions.

Plasmid-mediated efflux pumps are particularly important mechanisms of resistance to many antibiotics (85), metals (349), and cationic disinfectants and antiseptics such as QACs, chlorhexidine, diamidines, and acridines, as well as to ethidium bromide (239, 289, 324–336, 363–368). Recombinant *S. aureus* plasmids transferred into *E. coli* are responsible for conferring increased MICs of cationic agents to the gram-negative organism (505, 544). Midgley (324, 325) demonstrated that a plasmid-borne, ethidium resistance determinant from *S. aureus* cloned in *E. coli* encodes resistance to ethidium bromide and to QACs, which are expelled from the cells. A similar efflux system is present in *Enterococcus hirae* (326).

Sasatsu et al. (463) showed that duplication of *ebr* is responsible for resistance to ethidium bromide and to some antiseptics. Later, Sasatsu et al. (466) examined the origin of *ebr* (now known to be identical to *qacCD*) in *S. aureus*; *ebr* was found in antibiotic-resistant and -sensitive strains of *S. aureus*, CNS, and enterococcal strains. The nucleotide sequences of the amplified DNA fragment from sensitive and resistant strains were identical, and it was proposed that in antiseptic-resistant cells there was an increase in the copy number of the *ebr* (*qacCD*) gene whose normal function was to remove toxic substances from normal cells of staphylococci and enterococci.

Based on DNA homology, it was proposed that *qacA* and related genes carrying resistance determinants evolved from preexisting genes responsible for normal cellular transport systems (405) and that the antiseptic resistance genes evolved before the introduction and use of topical antimicrobial products and other antiseptics and disinfectants (288, 289, 365, 367, 368, 405).

Baquero et al. (23) have pointed out that for antibiotics, the presence of a specific resistance mechanism frequently contributes to the long-term selection of resistant variants under in vivo conditions. Whether low-level resistance to cationic antiseptics, e.g., chlorhexidine, QACs, can likewise provide a selective advantage on staphylococci carrying *qac* genes remains to be elucidated. The evidence is currently contentious and inconclusive.

(iii) Plasmid-mediated antiseptic and disinfectant resistance in other gram-positive bacteria. Antibiotic-resistant corynebacteria may be implicated in human infections, especially in the immunocompromised. 'Group JK' coryneforms (*Corynebacterium jeikeium*) were found to be more tolerant than other coryneforms to the cationic disinfectants ethidium bromide and hexachlorophene, but studies with plasmid-containing and plasmid-cured derivatives produced no evidence of plasmid-associated resistance (285). *Enterococcus faecium* strains showing high level resistance to vancomycin, gentamicin, or both are not more resistant to chlorhexidine or other nonantibiotic agents (9, 11, 20, 319). Furthermore, despite the extensive dental use of chlorhexidine, strains of *Streptococcus mutans* remain sensitive to it (235). To date, therefore, there is little or no evidence of plasmid-associated resistance of nonstaphylococcal gram-positive bacteria to antiseptics and disinfectants.

Mutational resistance to antiseptics and disinfectants. Chromosomal mutation to antibiotics has been recognized for decades. By contrast, fewer studies have been performed to determine whether mutation confers resistance to antiseptics and disinfectants. It was, however, demonstrated over 40 years ago (77, 78) that *S. marcescens*, normally inhibited by QACs at <100 μg/ml, could adapt to grow in 100,000 μg of a QAC per ml. The resistant and sensitive cells had different surface characteristics (electrophoretic mobilities), but resistance could be lost when the cells were grown on QAC-free media. One problem associated with QACs and chlorhexidine is the turbidity produced in liquid culture media above a certain concentration (interaction with agar also occurs), which could undoubtedly interfere with the determination of growth. This observation is reinforced by the findings presented by Nicoletti et al. (354).

Prince et al. (383) reported that resistance to chlorhexidine could be induced in some organisms but not in others. For example, *P. mirabilis* and *S. marcescens* displayed 128- and 258-fold increases, respectively, in resistance to chlorhexidine, whereas it was not possible to develop resistance to chlorhexidine in *Salmonella enteritidis*. The resistant strains did not show altered biochemical properties of changed virulence for mice, and some strains were resistant to the QAC benzalkonium chloride. Moreover, resistance to chlorhexidine was stable in *S. marcescens* but not in *P. mirabilis*. Despite extensive experimentation with a variety of procedures, Fitzgerald et al. (148) were unable to develop stable chlorhexidine resistance in *E. coli* or *S. aureus*. Similar observations were made by Cookson et al. (89), who worked with MRSA and other strains of *S. aureus*, and by McDonnell et al. (319), who worked with MRSA and enterococci. Recently, stable chlorhexidine resistance was developed in *P. stutzeri* (502); these strains showed

various levels of increased tolerance to QACs, triclosan, and some antibiotics, probably as a result of a nonspecific alteration of the cell envelope (452). The adaptation and growth of *S. marcescens* in contact lens disinfectants containing chlorhexidine, with cross-resistance to a QAC, have been described previously (166).

Chloroxylenol-resistant strains of *P. aeruginosa* were isolated by repeated exposure in media containing gradually increasing concentrations of the phenolic, but the resistance was unstable (432). The adaptation of *P. aeruginosa* to QACs is a well-known phenomenon (1, 2, 240). Resistance to amphoteric surfactants has also been observed, and, interestingly, cross-resistance to chlorhexidine has been noted (240). This implies that the mechanism of such resistance is nonspecific and that it involves cellular changes that modify the response of organisms to unrelated biocidal agents. Outer membrane modification is an obvious factor and has indeed been found with QAC-resistant and amphoteric compound-resistant *P. aeruginosa* (240) and with chlorhexidine-resistant *S. marcescens* (166). Such changes involve fatty acid profiles and, perhaps more importantly, outer membrane proteins. It is also pertinent to note here the recent findings of Langsrud and Sundheim (274). In this study, resistance of *P. fluorescens* to QACs was reduced when EDTA was present with the QAC (although the lethal effect was mitigated after the cells were grown in medium containing QAC and EDTA); similar results have been found with laboratory-generated *E. coli* mutants for which the MICs of triclosan were increased (318). EDTA has long been known (175, 410) to produce changes in the outer membrane of gram-negative bacteria, especially pseudomonads. Thus, it appears that, again, the development of resistance is associated with changes in the cell envelope, thereby limiting uptake of antiseptics and disinfectants.

Hospital (as for other environmental) isolates of gram-negative bacteria are invariably less sensitive to disinfectants than are laboratory strains (196, 209, 279, 286, 492). Since plasmid-mediated transfer has apparently been ruled out (see above), selection and mutation could play an important role in the presence of these isolates. Subinhibitory antibiotic concentrations may cause subtle changes in the bacterial outer structure, thereby stimulating cell-to-cell contact (109); it remains to be tested if residual concentrations of antiseptics or disinfectants in clinical situations could produce the same effect.

Another insusceptibility mechanism has been put forward, in this instance to explain acridine resistance. It has been proposed (270, 351) that proflavine-sensitive and -resistant cells are equally permeable to the acridine but that resistant cells possessed the ability to expel the bound dye. This is an important point and one that has been reinforced by more recent studies that demonstrate the significance of efflux in resistance of bacteria to antibiotics (284, 330, 355). Furthermore, multidrug resistance (MDR) is a serious problem in enteric and other gram-negative bacteria. MDR is a term used to describe resistance mechanisms used by genes that form part of the normal cell genome (168). These genes are activated by induction or mutation caused by some types of stress, and because they are distributed ubiquitously, genetic transfer is not needed. Although such systems are most important in the context of antibiotic resistance, George (168) provides several examples of MDR systems in which an operon or gene is associated with changes in antiseptic or disinfectant susceptibility; e.g., (i) mutations at an *acr* locus in the Acr system render *E. coli* more sensitive to hydrophobic antibiotics, dyes, and detergents; (ii) the *robA* gene is responsible for overexpression in *E. coli* of the RobA protein that confers multiple antibiotic and heavy-metal resistance (interestingly, Ag^+ may be effluxed [350]); and (iii) the MarA protein controls a set of genes (*mar* and *soxRS* regulons) that confer resistance not only to several antibiotics but also to superoxide-generating agents. Moken et al. (333) have found that low concentrations of pine oil (used as a disinfectant) could select for *E. coli* mutants that overexpressed MarA and demonstrated low levels of cross-resistance to antibiotics. Deletion of the *mar* or *acrAB* locus (the latter encodes a PMF-dependant efflux pump) increased the susceptibility of *E. coli* to pine oil; deletion of *acrAB*, but not of *mar*, increased the susceptibility of *E. coli* to chloroxylenol and to a QAC. In addition, the *E. coli* MdfA (multidrug transporter) protein was recently identified and confers greater tolerance to both antibiotics and a QAC (benzalkonium) (132). The significance of these and other MDR systems in bacterial susceptibility to antiseptics and disinfectants, in particular the issue of cross-resistance with antibiotics, must be studied further. At present, it is difficult to translate these laboratory findings to actual clinical use, and some studies have demonstrated that antibiotic-resistant bacteria are not significantly more resistant to the lethal (or bactericidal) effects of antiseptic and disinfectants than are antibiotic-sensitive strains (11, 88, 89, 319).

TABLE 12. Possible mechanisms of fungal resistance to antiseptics and disinfectants

Type of resistance	Possible mechanism	Example(s)
Intrinsic	Exclusion	Chlorhexidine
	Enzymatic inactivation	Formaldehyde
	Phenotypic modulation	Ethanol
	Efflux	Not demonstrated to date[a]
Acquired	Mutation	Some preservative
	Inducible efflux	Some preservatives[a]
	Plasmid-mediated responses	Not demonstrated to date

[a] Efflux is now known to be one mechanism of fungal resistance to antibiotics (531).

Mechanisms of Fungal Resistance to Antiseptics and Disinfectants

In comparison with bacteria, very little is known about the ways in which fungi can circumvent the action of antiseptics and disinfectants (104, 111, 296). There are two general mechanisms of resistance (Table 12): (i) intrinsic resistance, a natural property or development of an organism (201); and (ii) acquired resistance. In one form of intrinsic resistance, the cell wall presents a barrier to reduce or exclude the entry of an antimicrobial agent. The evidence to date is somewhat patchy, but the available information links cell wall glucan, wall thickness, and relatively porosity to the susceptibility of *Saccharomyces cerevisiae* to chlorhexidine (Table 13) (204–208). Protoplasts of this organism prepared by glucuronidase in the presence of β-mercaptoethanol are lysed by chlorhexidine concentrations well below those effective against "normal" (whole) cells. Furthermore, culture age influences the response of *S. cerevisiae* to chlorhexidine; the cells walls are much less sensitive at stationary phase than at logarithmic growth phase (208), taking up much less [^{14}C]chlorhexidine gluconate (206). Gale (165) demonstrated a phenotypic increase in the resistance of *Candida albicans* to the polyenic antibiotic amphotericin B as the organisms entered the stationary growth phase, which was attributed to cell wall changes involving tighter cross-linking (74). Additionally, any factor increasing glucanase activity increased amphotericin sensitivity.

The porosity of the yeast cell wall is affected by its chemical

TABLE 13. Parameters affecting the response of *S. cerevisiae* to chlorhexidine[a]

Parameter	Role in susceptibility of cells to chlorhexidine
Cell wall composition	
Mannan	No role found to date
Glucan	Possible significance: at concentrations below those active against whole cells, chlorhexidine lyses protoplasts
Cell wall thickness	Increases in cells of older cultures: reduced chlorhexidine uptake responsible for decreased activity(?)
Relative porosity	Decreases in cells of older cultures: reduced chlorhexidine uptake responsible for decreased activity(?)
Plasma membrane	Changes altering CHG susceptibility(?); not investigated to date

[a] Data from references 204 to 208 and 436.

composition, with the wall acting as a barrier or modulator to the entry and exit of various agents. DeNobel et al. (117–119) used the uptake of fluorescein isothiocyanurate (FITC) dextrans and the periplasmic enzyme invertase as indicators of yeast cell wall porosity. Intact *S. cerevisiae* cells were able to endocytose FITC dextrans of 70 but not of 150. A new assay for determining the relative cell wall porosity in yeast based upon polycation-induced leakage of UV-absorbing compounds was subsequently developed. Hiom et al. (206, 208) found that the relative porosity of cells decreases with increasing culture age and that there was a reduced uptake of radiolabeled chlorhexidine gluconate. As the age of an *S. cerevisiae* culture increases, there is a significant increase in the cell wall thickness, with values of 0.19, 0.25, and 0.31 μm recorded for cells from 1-, 2-, and 6-day old cultures, respectively (206).

These findings (Table 13) can provide a tentative picture of the cellular factors that modify the response of *S. cerevisiae* to chlorhexidine. Mannan mutants of *S. cerevisiae* show a similar degree of sensitivity to chlorhexidine as the parent strain (204). The glucan layer is shielded from β-glucuronidase by mannoproteins, but this effect is overcome by β-mercaptoethanol (119). The mannoprotein consists of two fractions, sodium dodecyl sulfate-soluble mannoproteins and sodium dodecyl sulfate-insoluble, glucanase-soluble ones: the latter limit cell wall porosity (119). Thus, glucan (and possibly mannoproteins) plays a key role in determining the uptake and hence the activity of chlorhexidine in *S. cerevisiae*. *C. albicans* is less sensitive and takes up less [^{14}C]chlorhexidine overall (206), but only a few studies with this organism and with molds have been performed.

Yeasts grown under different conditions have variable levels of sensitivity to ethanol (176, 402). Cells with linoleic acid-enriched plasma membranes are more resistant to ethanol than are cells with oleic acid-enriched ones, from which it has been inferred that a more fluid membrane enhances ethanol resistance (6).

There is no evidence to date of antiseptic efflux (although benzoic acid in energized cells is believed to be eliminated by flowing down the electrochemical gradient [529]) and no evidence of acquired resistance by mutation (except to some preservatives [436]) or by plasmid-mediated mechanisms (426, 436). It is disappointing that so few rigorous studies have been performed with yeasts and molds and antiseptics and disinfectants (see also Miller's [328] treatise on mechanisms for reaching the site of action). Molds are generally more resistant than yeasts (Table 14) and considerably more resistant than nonsporulating bacteria (Table 15). Mold spores, although more resistant than nonsporulating bacteria, are less resistant than bacterial spores to antiseptics and disinfectants (436). It is tempting to speculate that the cell wall composition in molds confers a high level of intrinsic resistance on these organisms.

Mechanisms of Viral Resistance to Antiseptics and Disinfectants

Early studies on the effects of disinfectants on viruses were reviewed by Grossgebauer (189). Potential viral targets are the viral envelope, which contains lipids and is a typical unit membrane; the capsid, which is principally protein in nature; and the genome. An important hypothesis was put forward in 1963 (258) and modified in 1983 (259) in which it was proposed that viral susceptibility to disinfectants could be based on whether viruses were "lipophilic" in nature, because they possessed a lipid envelope (e.g., herpes simplex virus [259]) or "hydrophilic" because they did not (e.g., poliovirus [514]). Lipid-enveloped viruses were sensitive to lipophilic-type disinfectants, such as 2-phenylphenol, cationic surfactants (QACs), chlorhexidine, and isopropanol, as well as to ether and chloroform. Klein and Deforest (259) further classified viruses into three groups (Table 16), A (lipid containing), B (nonlipid picornaviruses), and C (other nonlipid viruses larger than those in group B) and disinfectants into two groups, broad-spectrum ones that inactivated all viruses and lipophilic ones that failed to inactivate picornoviruses and parvoviruses.

Capsid proteins are predominantly protein in nature, and biocides such as glutaraldehyde, hypochlorite, ethylene oxide, and hydrogen peroxide, which react strongly with amino or sulfhydryl groups might possess virucidal activity. It must, however, be added that destruction of the viral capsid may result in the release of a potentially infectious nucleic acid and that viral inactivation would only be complete if the viral nucleic acid is also destroyed.

Unfortunately, the penetration of antiseptics and disinfectants into different types of viruses and their interaction with viral components have been little studied, although some information has been provided by investigations with bacteriophages (307). Bacteriophages are being considered as "indicator species" for assessing the virucidal activity of disinfectants (108) and could thus play an increasing important role in this context; for example, repeated exposure of *E. coli* phage f2 to chlorine was claimed to increase its resistance to disinfection (542).

Thurman and Gerber (509, 510) pointed out that conflicting results on the actions of disinfectants on different virus types were often reported, and they suggested that the structural integrity of a virus was altered by an agent that reacted with viral capsids to increase viral permeability. Thus, a "two-stage"

TABLE 14. Lethal concentrations of antiseptics and disinfectants toward some yeasts and molds[a]

Antimicrobial agent[b]	Lethal concn (μg/μl) toward:		
	Yeast (*Candida albicans*)	Molds	
		Penicillium chrysogenum	*Aspergillus niger*
QACs			
Benzalkonium chloride	10	100–200	100–200
Cetrimide/CTAB	25	100	250
Chlorhexidine	20–40	400	200

[a] Derived in part from data in reference 525.
[b] CTAB, cetyltrimethylammonium bromide.

TABLE 15. Kinetic approach: *D*-values at 20°C of phenol and benzalkonium chloride against fungi and bacteria[a]

Antimicrobial agent	pH	Concn (%, wt/vol)	*D*-value (h)[b] against:				
			Aspergillus niger	*Candida albicans*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Phenol	5.1	0.5	20	13.5	0.94	—[c]	0.66
	6.1	0.5	32.4	18.9	1.72	0.17	1.9
Benzalkonium chloride	5.1	0.001	—[d]	9.66	0.06	3.01	3.12
	6.1	0.002	—[d]	5.5	—[c]	0.05	0.67

[a] Abstracted from the data in references 244 and 245.
[b] *D*-values are the times to reduce the viable population by 1 log unit.
[c] Inactivation was so rapid that the *D*-values could not be measured.
[d] No inactivation: fungistatic effect only.

disinfection system could be an efficient means of viral inactivation while overcoming the possibility of multiplicity reactivation (first put forward by Luria [293]) to explain an initial reduction and then an increase in the titer of disinfectant-treated bacteriophage. Multiplicity reactivation as a mechanism of resistance was supported by the observation of Young and Sharp (546) that clumping of poliovirus following partial inactivation by hypochlorite significantly increased the phage titer. It is envisaged as consisting of random damage to the capsid protein or nucleic acid of clumped, noninfectious virions from which complementary reconstruction of an infectious particle occurs by hybridization with the gene pool of the inactivated virions (298).

Another resistance mechanism also involves viral aggregation, e.g., the persistence of infectivity of formaldehyde-treated poliovirus (458) and the resistance of Norwalk virus to chlorination (249). A typical biphasic survival curve of enterovirus and rotavirus exposed to peracetic acid is also indicative of the presence of viral aggregates (198).

Finally, there remains the possibility of viral adaptation to new environmental conditions. In this context, Bates et al. (28) described the development of poliovirus having increased resistance to chlorine inactivation. Clearly, much remains to be learned about the mechanism of viral inactivation by and viral resistance to disinfectants.

Mechanisms of Protozoal Resistance to Antiseptics and Disinfectants

Intestinal protozoa, such as *Cryptosporidium parvum*, *Entamoeba histolytica*, and *Giardia intestinalis*, are all potentially pathogenic to humans and have a resistant, transmissible cyst (or oocyst for *Cryptosporidium*) (233, 234). Of the disinfectants available currently, ozone is the most effective protozoan cysticide, followed by chlorine dioxide, iodine, and free chlorine, all of which are more effective than the chloramines (234, 264). Cyst forms are invariably the most resistant to chemical disinfectants (Fig. 1). The reasons for this are unknown, but it would be reasonable to assume that cysts, similar to spores, take up fewer disinfectant molecules from solution than do vegetative forms.

Some recent studies have compared the responses of cysts and trophozoites of *Acanthamoeba castellanii* to disinfectants used in contact lens solutions and monitored the development of resistance during encystation and the loss of resistance during excystation (251–255). The lethal effects of chlorhexidine and of a polymeric biguanide were time and concentration dependent, and mature cysts were more resistant than preencystment trophozoites or preexcystment cysts. The cyst "wall" appeared to act as a barrier to the uptake of these agents, thereby presenting a classical type of intrinsic resistance mechanism (163). Acanthamoebae are capable of forming biofilms on surfaces such as contact lenses (186). Although protozoal biofilms have yet to be studied extensively in terms of their response to disinfectants, it is apparent that they could play a significant role in modulating the effects of chemical agents.

Mechanisms of Prion Resistance to Disinfectants

The transmissible degenerative encephalopathies (TDEs) form a group of fatal neurological diseases of humans and other animals. TDEs are caused by prions, abnormal proteinaceous agents that appear to contain no agent-specific nucleic acid (385). An abnormal protease-resistant form (PrP^{res}) of a normal host protein is implicated in the pathological process.

Prions are considered highly resistant to physical and chemical agents (Fig. 1), although the fact that crude preparations are often studied means that extraneous materials could, at least to some extent, mask the true efficacy of these agents (503). According to Taylor (503), there is currently no known decontamination procedure that will guarantee the complete absence of infectivity in TDE-infected tissues processed by histopathological procedures. Prions survive acid treatment, but a synergistic effect with autoclaving plus sodium hydroxide treatment is observed. Formaldehyde, unbuffered glutaraldehyde (acidic pH), and ethylene oxide have little effect on infectivity, although chlorine-releasing agents (especially hypochlorites), sodium hydroxide, some phenols, and guanidine thiocyanate are more effective (141, 309, 503).

With the information presently available, it is difficult to explain the extremely high resistance of prions, save to comment that the protease-resistant protein is abnormally stable to degradative processes.

CONCLUSIONS

It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported. Of the mechanisms that have been studied, the most significant are clearly intrinsic, in particular the ability to sporulate, adaptation of pseudomonads, and the protective effects of biofilms. In these cases, "resistance" may be incorrectly used and "tolerance," defined as developmental or protective effects that permit microorganisms to survive in the presence of an active agent, may be more correct. Many of these reports of resistance have often paralleled issues including inadequate cleaning, incorrect product use, or ineffective infection control practices, which cannot be underestimated. Some acquired mechanisms (in particular with heavy-metal resistance) have also been shown to be clinically significant, but in most cases the results have been spec-

TABLE 16. Viral classification and response to some disinfectants[a]

Viral group	Lipid envelope[b]	Examples of viruses	Effects of disinfectants[c]	
			Lipophilic	Broad-spectrum
A	+	HSV, HIV, Newcastle disease virus, rabies virus, influenza virus	S	S
B	−	Non-lipid picornaviruses (poliovirus, Coxsackie virus, echovirus)	R	S
C	−	Other larger nonlipid viruses (adenovirus, reovirus)	R	S

[a] Data from reference 259; see also reference 444. For information on the inactivation of poliovirus, see reference 514.
[b] Present (+) or absent (−).
[c] Lipophilic disinfectants include QACs and chlorhexidine. S, sensitive; R, resistant.

ulative. Increased MICs have been confirmed, in particular for staphylococci. However, few reports have further investigated increased bactericidal concentrations or actual use dilutions of products, which in many cases contain significantly higher concentrations of biocides, or formulation attributes, which can increase product efficacy; in a number of cases, changes in the MICs have actually been shown not to be significant (9, 88, 89, 319, 428). Efflux mechanisms are known to be important in antibiotic resistance, but it is questionable if the observed increased MICs of biocides could have clinical implications for hard-surface or topical disinfection (423, 428). It has been speculated that low-level resistance may aid in the survival of microorganisms at residual levels of antiseptics and disinfectants; any possible clinical significance of this remains to be tested. With growing concerns about the development of biocide resistance and cross-resistance with antibiotics, it is clear that clinical isolates should be under continual surveillance and possible mechanisms should be investigated.

It is also clear that antiseptic and disinfectant products can vary significantly, despite containing similar levels of biocides, which underlines the need for close inspection of efficacy claims and adequate test methodology (183, 423, 428). In addition, a particular antiseptic or disinfectant product may be better selected (as part of infection control practices) based on particular circumstances or nosocomial outbreaks; for example, certain active agents are clearly more efficacious against gram-positive than gram-negative bacteria, and *C. difficile* (despite the intrinsic resistance of spores) may be effectively controlled physically by adequate cleaning with QAC-based products.

In conclusion, a great deal remains to be learned about the mode of action of antiseptics and disinfectants. Although significant progress has been made with bacterial investigations, a greater understanding of these mechanisms is clearly lacking for other infectious agents. Studies of the mechanisms of action of and microbial resistance to antiseptics and disinfectants are thus not merely of academic significance. They are associated with the more efficient use of these agents clinically and with the potential design of newer, more effective compounds and products.

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Exhibit H

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Improved Inactivation of Nonenveloped Enteric Viruses and Their Surrogates by a Novel Alcohol-Based Hand Sanitizer▽

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Norovirus is the leading cause of food-related illness in the United States, and contamination of ready-to-eat items by food handlers poses a high risk for disease. This study reports the in vitro (suspension test) and in vivo (fingerpad protocol) assessments of a new ethanol-based hand sanitizer containing a synergistic blend of polyquaternium polymer and organic acid, which is active against viruses of public health importance, including norovirus. When tested in suspension, the test product reduced the infectivity of the nonenveloped viruses human rotavirus (HRV), poliovirus type 1 (PV-1), and the human norovirus (HNV) surrogates feline calicivirus (FCV) F-9 and murine norovirus type 1 (MNV-1) by greater than 3 $\log_{10}$ after a 30-s exposure. In contrast, a benchmark alcohol-based hand sanitizer reduced only HRV by greater than 3 $\log_{10}$ and none of the additional viruses by greater than 1.2 $\log_{10}$ after the same exposure. In fingerpad experiments, the test product produced a 2.48 $\log_{10}$ reduction of MNV-1 after a 30-s exposure, whereas a 75% ethanol control produced a 0.91 $\log_{10}$ reduction. Additionally, the test product reduced the infectivity titers of adenovirus type 5 (ADV-5) and HRV by ≥3.16 $\log_{10}$ and ≥4.32 $\log_{10}$, respectively, by the fingerpad assay within 15 s; and PV-1 was reduced by 2.98 $\log_{10}$ in 30 s by the same method. Based on these results, we conclude that this new ethanol-based hand sanitizer is a promising option for reducing the transmission of enteric viruses, including norovirus, by food handlers and care providers.

Proper and frequent hand hygiene is crucial for infection control (10, 25). Whereas hand washing with soap and water remains common and relevant, influential organizations such as the U.S. Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) now recommend alcohol-based hand sanitizers (ABHS) for hand hygiene when hands are not visibly soiled (10, 42). Among the advantages of ABHS over traditional soap-and-water washing are (i) a faster microbial kill, (ii) a greater reduction in microbial load, (iii) a broader spectrum of microbicidal activity, (iv) relative ease of use and time savings, (v) better skin tolerance in spite of frequent use, (vi) convenience and freedom from dependence on sinks and running water, and (vii) water conservation (7–10, 38, 42). These factors, together with evidence for higher levels of compliance with hand hygiene and reduced rates of certain types of hospital-associated infections, have promoted wide acceptance of ABHS in health care and moderate acceptance in food industries (7, 10, 19, 37).

While improving compliance with hand hygiene in the food service and food processing industries remains a challenge, the presence of nonenveloped viruses, which are more difficult to inactivate by commonly used hand sanitizers, adds another layer of concern. Statistics from the Foodborne-Disease Outbreak Surveillance System (1998 to 2002) indicate that viral pathogens (predominantly norovirus) accounted for 33% of outbreaks and 41% of cases of infection with known etiology (27). The annual number of food-related infections in the United States is estimated at 76 million, with viruses accounting for 79% and human norovirus (HNV) alone accounting for 59% of such cases (30). The 2005 Food Code, which regulates hand hygiene in the food service sector, prescribes hand washing as the primary means for hand decontamination, with the use of ABHS only as an adjunct to hand washing (37). It is well documented that ethanol alone can rapidly inactivate vegetative bacteria, fungi, and enveloped viruses, but its activity against nonenveloped viruses varies more widely (10). There remains a need for hand sanitizers with demonstrated activity against a broad spectrum of nonenveloped viruses, including HNV.

Because routine culture and infectivity assays of HNV are not possible, HNV surrogates are routinely used to evaluate the virucidal activity of disinfectants and antiseptics (6, 13). Feline calicivirus (FCV), one such surrogate, survives well in the environment and is relatively resistant to chemical challenges (13, 14, 16, 28). However, since FCV is primarily a respiratory pathogen and appears to be less acid tolerant than HNV, its relevance as a surrogate for HNV has been questioned by some investigators (11). The recent in vitro cultivation of murine norovirus type 1 (MNV-1) provides an alternative and perhaps a more suitable surrogate than FCV (11, 40, 41).

This study presents findings of the in vitro and in vivo activities of a new synergistically formulated ABHS against several nonenveloped viruses, including surrogates for HNV.

MATERIALS AND METHODS

Antimicrobial test articles. Purell VF447 (GOJO Industries Inc., Akron, OH), the test product used in this study, contains 70% ethanol (vol/vol) as the active ingredient and is formulated with a synergistic blend of polyquaternium-37 (PQ-37) and citric acid, as well as gelling and skin conditioning ingredients (M. Snyder, D. R. Macinga, and J. W. Arbogast, U.S. patent application 11/499,227.).

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TABLE 1. Mammalian viruses and corresponding cell lines used in this study

Virus (ATCC strain)	Host cell (ATCC strain)
Adenovirus type 2 (VR-846)	A-549 (CCL-185)
Adenovirus type 5 (VR-1516)	293 (CRL-1573); Vero (CCL-81)
Feline calicivirus, F-9 (VR-782)	CrFK (CCL-94)
Hepatitis A virus, HM-175 (VR-2093)	FRhK-4 (CRL-1688)
Murine norovirus type 1, P3[a]	RAW 264.7 (TIB-71)
Poliovirus type 1 Sabin (VR-1000)	Vero (CCL-81)
Human rotavirus, Wa (VR-2018)	MA-104 (CRL-2378.1)

[a] A gift from H. W. Virgin, Washington University, St. Louis, MO.

The benchmark hand sanitizer, Purell Instant Hand Sanitizer (GOJO), contains 62% ethanol (vol/vol) as the active ingredient. The 75% ethanol (vol/vol) reference solution was prepared in distilled water.

Bacteriophage MS2 suspension assays. MS2 was used as the surrogate for nonenveloped mammalian viruses to screen for compounds with virucidal activity. A 0.1-ml aliquot of MS2 (ATCC 15597-B1) suspension was added to 9.9 ml of the test substance. After a 60-s contact time at room temperature with mixing, a 0.1-ml volume was removed and neutralized by dilution in 9.9 ml of Dey-Engley neutralizing broth. Further 10-fold serial dilutions were prepared in Dey-Engley broth. The viable phage remaining were quantified by infecting *Escherichia coli* (ATCC 15597) by using the soft agar technique (20).

Viruses, cell lines, and viral propagation. The mammalian viruses used and their respective host cell lines are listed in Table 1. Human adenovirus type 5 (ADV-5; ATCC VR-1516) was grown in 293 cells (ATCC CRL-1573) and enumerated by plaque assay in Vero cells (ATCC CCL-81) because 293 cells yielded higher titers of the virus and Vero cell monolayers proved more stable for the 7 to 8 days of incubation required for the plaque assay. All other viruses were grown and plaque assayed using the appropriate cell line as indicated in Table 1. The procedures for growing cell monolayers and for preparing virus pools have been described previously (6, 32).

Infectivity assays. To determine the infectious titer of MNV-1 in virucidal suspension assays, conventional plaque assay techniques were employed as previously reported (11). Briefly, RAW 264.7 cells were dispensed in 60-mm-diameter cell culture plates at a density of 2×10^6 cells per plate and grown to 80 to 90% confluence in 5 ml of complete minimum essential medium at 37°C. Cell monolayers were infected with 200 μl of a 10-fold dilution series of the virus for 1 h at 37°C and, after the inoculum was removed, the cells were overlaid with 5 ml of medium containing 0.5% agarose and incubated for 48 h. A second agarose overlay, which included 0.75% neutral red solution (Sigma-Aldrich), was then added (3 ml), and plaques were counted at 5 to 8 h thereafter. Plates with 5 to 50 plaques were used to determine the virus titer in PFU. Infectivity assays for all other viruses in suspension assays were done by measuring the residual infectious virus detected by the virus-induced cytopathic effect (26). Viral titers were expressed as the $-\log_{10}$ of the 50% titration endpoint for infectivity ($TCID_{50}$) per 0.1 ml as calculated by the method of Spearman-Kärber (21).

Infectivity assays for fingerpad experiments were done by plaque assay using a slightly different method from that described above. Host cell monolayers were prepared in 12-well cell culture plates (Corning, Ithaca, NY). Each well received 2 ml of cell suspension in growth medium and was incubated at 37°C with 5% CO_2 until cells were confluent. Samples for assay were serially diluted in Earle's balanced salt solution (EBSS; Invitrogen, Burlington, ON, Canada). Cell monolayers were infected with 100 μl of each serial dilution and incubated at 37°C in 5% CO_2 for 90 min with gentle rocking every 30 min. Two milliliters of warm (45°C) sterile agar overlay was placed in each well, and the plates were incubated at 37°C in 5% CO_2 until plaque formation. Thereafter, monolayers were fixed in 2 ml of 3.7% formaldehyde (Anachemia; Lachine, QC, Canada) in normal saline for at least 4 h. Formaldehyde was discarded, and agar plugs were removed by holding the plates under running warm tap water. The fixed cells were then stained with 0.1% (wt/vol) aqueous crystal violet solution for 20 min (6). Plaques were then counted, and their numbers were recorded.

Each plaque assay plate included at least two wells which served as negative controls to make sure that the cell monolayers were not contaminated. One well was used as a positive control to ensure the presence of infectious virus and its ability to infect the host cells used in the assay. An assay was regarded as valid only if the negative controls remained intact and there was detectable degeneration of the cells in the positive control (33).

Suspension assays. Suspension tests for virucidal activity were performed using a modification of ASTM standard E-1052 (3). A 4.5-ml sample of the test substance was dispensed into a sterile 15-ml conical tube and mixed with 0.5 ml of the virus suspension. The mixture was vortexed for 10 s and held for the remainder of the 30-s exposure time. Immediately following the exposure period, a 0.1-ml aliquot was removed from the tube, neutralized by 10-fold serial dilution into minimum essential medium containing 10% fetal bovine serum and titrated for the presence of viable virus by infectivity assay. For MNV-1, infectivity was determined by plaque assay. In instances where residual cytotoxicity was noted after exposure to a test substance, the sample was removed in accordance with the column chromatography method recommended by ASTM standard E-1482, prior to plaque assay (4). For all other viruses, infectivity was determined by virus-induced cytopathic effect, and $\log_{10}$ reductions were calculated by subtracting the $TCID_{50}$ of the test treatment from the $TCID_{50}$ of the virus control (21, 26). Consistent with recommendations of ASTM standard E-1052 (3), virus controls, neutralization controls, and cytotoxicity controls were also performed.

Fingerpad method. The fingerpad assay used in this study was based on ASTM standard E-1838-02, which has been described in detail previously (2, 32, 33). Prior to the initiation of the study, we obtained formal approval from the Ottawa Hospital Research Ethics Board. Briefly, each potential adult panelist was screened, using a set of inclusion/exclusion criteria, briefed on the objective and procedures for the study, and requested to review and sign an informed consent form. Just before their fingerpads were contaminated, the panelist thoroughly washed and dried his/her hands, using a nonmedicated liquid soap to remove any dirt and oil from the skin. To reduce any transient microorganisms on the panelist's hands, about 3.0 ml of 75% (vol/vol) ethanol was then dispensed onto the panelist's cupped palms, and he/she rubbed the alcohol over the entire surface of both hands until the liquid had evaporated completely.

Each virus to be tested was suspended in a soil load to simulate the presence of body secretions or excretions. The two types of soil loads, as permitted by ASTM standard E-1838-02 (2), were 5% bovine serum (final concentration) and a mixture of three components (tryptone, bovine serum albumin, and bovine mucin), with a total protein content roughly equal to that in 5% bovine serum (33).

To determine the number of PFU of the test virus placed onto each fingerpad, we placed 10 μl of the inoculum on each of the panelist's thumb pads and immediately eluted the pads ("time-zero control") as described below; the thumb pads were immediately decontaminated by pressing them for 2 to 3 min against a paper towel soaked in either 75% ethanol (for AD and HRV) or a 1:10 dilution of domestic bleach (for MNV-1, poliovirus [PV], and hepatitis A virus [HAV]). Each fingerpad was then contaminated with 10 μl of the test virus suspension, and the inocula were allowed to become visibly dry under ambient conditions, which required 20 to 25 min. To assess the loss in virus infectivity due to drying, one randomly selected fingerpad was eluted immediately upon drying, and another was eluted at the end of the experiment; the average level of infectious virus on these two fingerpads constituted the "baseline control."

The dried inocula on at least two randomly selected fingerpads (one on each hand) were then each exposed simultaneously to 1 ml of the test formulation contained in a 2-ml cryovial (Sarstedt Inc., Montreal, QC, Canada) for the desired contact time. The material remaining on each treated fingerpad was eluted with 1 ml of EBSS containing 0.1% (vol/vol) Tween-80 (BioShop, Burlington, ON, Canada). The degree of virus inactivation was determined by subjecting the eluates, from the control and the treated digits, to a plaque assay. Additional controls were included to determine (i) the level of infectious virus in the suspensions for the contamination of hands, (ii) any interference to virus infectivity that subcytotoxic levels of the test formulations might cause, and (iii) the absence of extraneous viral or bacterial contamination in the host cells (33). These controls ensured that a decreased (or lack of) recovery of virus after exposure to the test substance was due to inactivation by the test formulation.

Statistical considerations. For comparing the $\log_{10}$ reductions of a minimum of six replicates for various test articles against MS2, a one-way analysis of variance (ANOVA) with a Bonferroni post hoc analysis for multiple comparisons was performed at an alpha level of 0.05. Statistical comparisons of VF447 and the 75% ethanol control activities against MNV-1 were evaluated with Student's t test ($\alpha = 0.05$). Statistical analyses were conducted with GraphPad InStat version 3.06 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Identification of ethanol potentiators. Previous reports, as well as work in our laboratories, have demonstrated that ethanol possesses relatively weak activity against certain nonen-

TABLE 2. In vitro inactivation of MS2 after 60-s exposure to various alcohol mixtures

Active ingredient	Potentiator (% wt/wt)	MS2 $\log_{10}$ reduction
NaOCl (ppm)		
100	None	≥5.85
Ethanol (%)		
62	None	−0.10
70	None	−0.02
78	None	0.16
78	Benzethonium chloride (0.5%)	0.34
78	Chlorhexidine gluconate (2%)	0.48
78	Copper gluconate (0.04%)[a]	0.18
78	Silver zeolite (0.24%)[b]	0.58

[a] 60 ppm copper.
[b] 60 ppm silver.

veloped viruses, including PV and the HNV surrogate FCV (5, 13, 29). A suspension assay with bacteriophage MS2 was used to identify compounds capable of potentiating the activity of ethanol against nonenveloped viruses. MS2 was chosen because it has been demonstrated to be a good model for evaluating hand hygiene products which target nonenveloped viruses and because it is safe and relatively simple to handle (20).

Table 2 shows that MS2 is highly resistant to ethanol, which exhibited minimal activity in concentrations of up to 78%. In contrast, viable MS2 became undetectable after a 60-s exposure to 100 ppm of sodium hypochlorite (≥5.85 $\log_{10}$ reduction). Various compounds with known antimicrobial activity (including the topical antimicrobials chlorhexidine gluconate and benzethonium chloride and the transition metals copper and silver) produced small but measurable increases in the activity of ethanol. A large number of additional compounds did not improve the activity of ethanol against MS2 (data not shown). Interestingly, the addition of either citric acid or the cationic polymer PQ-37 each increased the activity of 78% ethanol against MS2 (Fig. 1). Statistical analysis using one-way ANOVA revealed that the increase in activity against MS2 resulting from the addition of PQ-37 to ethanol was significant ($P < 0.001$), whereas the increase from the addition of citric acid to ethanol was not statistically significant. Surprisingly, the combination of ethanol, PQ-37, and citric acid was synergistic against MS2, resulting in a reduction (4.36 $\log_{10}$) that was significantly higher than that of ethanol in combination with either citric acid (0.60 $\log_{10}$) or with PQ-37 (1.06 $\log_{10}$) alone ($P < 0.001$). Similar results were observed at lower ethanol concentrations, although the overall $\log_{10}$ reductions were lower (data not shown).

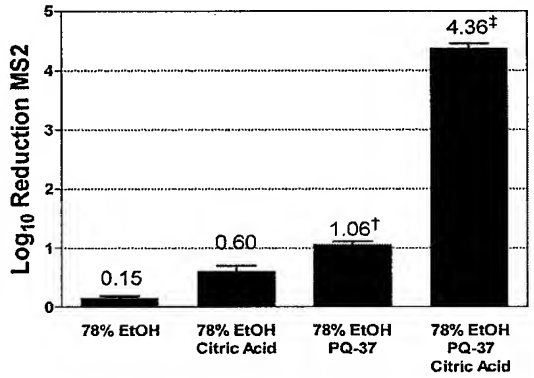

FIG. 1. Virucidal activity of various alcoholic mixtures as measured by a suspension test against MS2, with a 60-s exposure at room temperature. When they were included, citric acid was at 0.25% (wt/wt), and PQ-37 was at 0.4% (wt/wt). The bar heights indicate the mean values from a minimum of six replicates, and the error bars indicate standard errors of the means. † indicates significantly greater activity than 78% ethanol (EtOH) by one way ANOVA ($P < 0.001$). ‡ indicates significantly greater activity than all other alcoholic mixtures ($P < 0.001$).

TABLE 3. In vitro inactivation of MS2 after 60-s exposure to products with different active ingredients

Product	Active ingredient(s)	MS2 $\log_{10}$ reduction
Benchmark hand sanitizer	62% ethanol	0
VF447	70% ethanol	1.91
A	71% ethanol	0
B	95% ethanol	0.90
C	54.1% ethanol, 10% 1-propanol, 5.9% 1,2-propanediol, 5.7% 1,3-butanediol	1.79
D	70% ethanol, 0.1% 1,3-butanediol	0
E	70% isopropanol	0.05
F	75% isopropanol	0.18
G	45% isopropanol, 30% 1-propanol, 0.2% mecetronium etilsulfate	0

Based on the above results, a hand sanitizer was formulated for optimized virucidal performance, skin compatibility, and aesthetics. The formulation (termed Purell VF447 and hereafter referred to as VF447) contains 70% ethanol, PQ-37, citric acid, hydroxypropyl cellulose, and multiple skin-conditioning ingredients. Table 3 compares the in vitro virucidal activity of VF447 to that of several commercially available hand sanitizers with differing active ingredients against the MS2 surrogate after a 60-s exposure. Products containing up to 95% ethanol, up to 75% isopropanol, or mixtures of alcohols exhibited minimal virucidal activity against MS2. Only VF447 and the product based on a blend of alcohols and diols (23) inactivated MS2 by >1 $\log_{10}$ (1.91 $\log_{10}$ and 1.79 $\log_{10}$, respectively).

TABLE 4. In vitro comparison of benchmark sanitizer activity compared to that of VF447

Virus	Virus $\log_{10}$ reduction[a]	
	Benchmark hand sanitizer	VF447
Feline calicivirus	1	≥4.75
Murine norovirus	1.16	≥3.68
Adenovirus type 2	0.5	2.75
Hepatitis A virus	0	1.75
Poliovirus type 1	0	3.5
Rotavirus	≥5.75	≥4.75

[a] Values indicate reduction after a 30-s exposure. ≥ indicates inactivation values below that of the detection limit of the assay.

TABLE 5. In vivo efficacy of 75% ethanol against murine norovirus compared to that of VF447

Treatment	Exposure time (s)	No. of fingerpads examined	Mean $\log_{10}$ reduction	SD[b]	*P* value
VF447[a]	30	16	2.48	0.45	<0.0001
75% ethanol	30	8	0.91	0.57	

[a] Values shown are the combined results of two individual lots.
[b] SD, standard deviation.

Virucidal activity of the test product against nonenveloped enteric viruses by suspension assays. Table 4 shows a comparison of the virucidal activity of VF447 with that of a benchmark hand sanitizer (Purell Instant Hand Sanitizer; 62% ethanol) against several nonenveloped enteric viruses and their surrogates. The activity of the benchmark sanitizer varied considerably depending on the virus, and ranged from no reduction of HAV to >5 $\log_{10}$ reduction for human rotavirus (HRV) after a 30-s contact. The activity of VF447 was substantially higher for the ethanol-resistant nonenveloped viruses, reducing the infectivity of HAV, PV, and ADV-2 by 1.75 $\log_{10}$, 3.5 $\log_{10}$, and 2.75 $\log_{10}$, respectively. Of note was the improvement in virucidal activity against the HNV surrogates FCV (≥4.7 $\log_{10}$ reduction) and MNV-1 (≥3.68 $\log_{10}$ reduction). In each case, no infectious virus was detectable after a 30-s exposure to VF447, whereas the benchmark product produced only marginal reductions of the HNV surrogates (approximately 1 $\log_{10}$) after the same exposure time.

Inactivation of MNV-1 on fingerpads. Table 5 shows a comparison of the activity of VF447 with that of a 75% ethanol control against MNV-1 on the fingerpads of adults, using ASTM standard E-1838-02. The test product achieved an average reduction of 2.48 $\log_{10}$ in 30 s, which was significantly greater than that achieved by the 75% ethanol control (0.91 $\log_{10}$) at the same exposure time ($P < 0.0001$).

Inactivation of additional nonenveloped viruses on fingerpads. VF447 was further evaluated against additional nonenveloped enteric viruses, utilizing the fingerpad method (Table 6). Experiments using a 30-s contact resulted in no detectable recovery of ADV-5 and HRV from the majority of the fingerpads exposed to VF447. Baseline, input, and plaque interference controls (described in Materials and Methods) demonstrated that this was due to the inactivation of virus to a level below that of the detection limit of the assay (33). The average reduction values were ≥3.12 $\log_{10}$ and ≥3.84 $\log_{10}$ for ADV-5 and HRV, respectively. The test product was slightly less effective against PV (2.98 $\log_{10}$ reduction) and demonstrated lower activity against HAV (1.32 $\log_{10}$ reduction) after a 30-s exposure.

Because the test product reduced ADV-5 and HRV levels to below those of the detection limits after 30 s, additional experiments were conducted using a 15-s contact. Again, no virus was recovered from the majority of the fingerpads exposed to the test product, resulting in average estimated reduction values of ≥3.16 $\log_{10}$ and ≥4.32 $\log_{10}$ for ADV-5 and HRV, respectively.

TABLE 6. In vivo efficacy of VF447 against five nonenveloped mammalian viruses

Virus	Exposure time (s)	No. of fingerpads examined	Mean $\log_{10}$ reduction[a]	SD[b]
Adenovirus type 5[c]	15	8	≥3.16	0.12
	30	12	≥3.12	0.43
Rotavirus[c]	15	12	≥4.32	0.06
	30	12	≥3.84	0.35
Poliovirus type 1	30	12	2.98	0.50
Hepatitis A virus	30	2	1.32	NA

[a] ≥ indicates that no viruses were recovered from the treated fingerpads, values were calculated from baseline controls.
[b] SD, standard deviation; NA, not applicable.
[c] Values represent combined results of two individual lots.

DISCUSSION

This broad, multilaboratory study describes the antiviral performance of a new ethanol-based hand sanitizer optimized for activity against nonenveloped enteric viruses. The antiviral activity of the test product, VF447, was evaluated by both in vitro and in vivo methodologies at multiple time points against a broad set of nonenveloped enteric viruses and their surrogates. Overall, VF447 demonstrated in vitro virucidal activity that was superior to that of the benchmark sanitizer (Table 4) and those of a variety of commercial ABHS (Table 3) and performed statistically better than a 75% ethanol control solution, using in vivo fingerpad assays (Table 5).

The test product, VF447, was formulated with ethanol, which is recognized by the U.S. Food and Drug Administration as a safe and efficacious active ingredient for health care antiseptic drug products (15). An activity level of 70% was chosen since this is the maximum level of compliance with international food code regulations. PQ-37 and citric acid were included in VF447 because the combination of these two ingredients with ethanol was found to be synergistic, providing the greatest overall enhancement of virucidal action (Fig. 1). Additional experiments demonstrated that PQ-37 could be substituted with alternative polyquaterniums to produce similar results (Snyder et al., U.S. patent application 11/499,227.). Polyquaterniums are typically poor microbicides, and in fact, PQ-37 was found to have little microbicidal activity by itself. While the mechanism by which organic acid and polyquaterniums potentiate the activity of ethanol remains poorly understood, preliminary studies suggest that charge density plays a role (D. R. Macinga and J. W. Arbogast, unpublished data).

The sensitivity of FCV to low pH has been recently reported (11), and the acid-labile nature of rhinoviruses is also well known (18, 24, 36). Our findings show that the potentiation of the virucidal activity of ethanol by the addition of organic acids is virus specific. For example, when comparing similar formulations, we typically observed greater activity against HAV when acid was absent (D. R. Macinga and J. W. Arbogast, unpublished data). Overall, it appears that the addition of PQ-37 and citric acid uniquely maximizes the activity against mammalian nonenveloped viruses while still meeting the critical skin care performance and aesthetic requirements accepted in actual use.

The enteric viruses and their surrogates selected for this

study represent the majority of food-borne viral pathogens. As stated previously, HNV is the most epidemiologically important virus in food settings, followed closely by HAV (17). Rotaviruses have also been occasionally incriminated in food-borne outbreaks (1). The PV used here is prototypical of human enteroviruses and is often used in assessing antiseptics and disinfectants for their activities against viruses (e.g., European virucidal test norm, EN 14476 [14a]). While ADV-40 and -41 are incriminated in cases of gastroenteritis, they remain relatively difficult to work with in the laboratory. In view of this, ADV-2 and ADV-5 were used as surrogates for them in this study. It should be noted that the transmission of these enteric viruses is not limited to food settings. For example, HNV has plagued the cruise line industry for years, and its growing importance in long-term-care facilities has been documented recently (12, 39).

To our knowledge, this is the first published study that uses MNV-1 as a surrogate for HNV for the fingerpad method. The literature suggests that MNV-1 provides a more relevant surrogate because it belongs to the same genus as that of HNV and is also transmitted by the fecal-oral route in its natural host (22, 41). Nevertheless, because of the high relevance of HNV and the inability to assay the virus by standard means, both FCV and MNV-1 were used as surrogates for HNV in this study. The two surrogates were found to exhibit similar sensitivities to the products tested (Table 4). Whereas the benchmark sanitizer reduced each surrogate by approximately 1 $\log_{10}$ in 30 s, VF447 reduced both surrogates to a level below the detection limit in 30 s. These results confirm previous studies demonstrating the ethanol resistance of FCV and the suitability of MNV as an HNV surrogate when ethanol-based products are evaluated. Furthermore, the results demonstrate that antiviral activity is not dictated entirely by ethanol level and illustrates the benefits of the VF447 formulation.

Methodology challenges, limitations, and choices with hand hygiene product efficacy testing are typically a source of debate and discussion. The 15- and 30-s contact times evaluated in this study are highly relevant, as ABHS typically take about 15 to 30 s to dry, depending primarily on the amount applied to the hands. In previous studies that evaluated ABHS by the fingerpad method, contact times ranged from 20 to 60 s (23, 32). It can be argued that a 60-s contact time is not relevant to the end user; however, a 60-s contact time may have some scientific value (e.g., when comparing soil types and load effects). In addition to more testing at 15 s or less, future studies using actual HNV and intervention epidemiology studies to assess real-world infection rate outcomes would be highly valuable.

In this study, the initial screening with bacteriophage MS2 proved to be a useful tool for predicting the virucidal activity of test formulations against mammalian nonenveloped viruses. The relevance of in vitro suspension assays can be argued and should not be the sole means for evaluating the efficacy of hand hygiene products. They are, however, a critical tool in screening and ranking test articles because they afford major cost and time savings compared to that of in vivo testing. The fingerpad protocol is currently the best method to predict the real-world efficacy of hand hygiene products against viruses and has been shown to be comparable with whole-hand methods (35). In this set of experiments, the virucidal activity observed for VF447 in the suspension assays was a reliable predictor of in vivo activity measured by fingerpad experiments (e.g., the high activity of VF447 against the HNV surrogates by both methods). In conclusion, the use of MS2 for quick screening of technologies, in vitro suspension assays for comparing multiple products at multiple time points, and the fingerpad method for final confirmation were approaches that were both efficient and effective.

The results of this study demonstrate that VF447 is a promising adjunct to hand washing for use in multiple settings, including the food service and food processing industries. Previous work has demonstrated that the combination of hand washing and hand sanitizing produces greater reductions in bacterial levels than either practice alone (31). Future studies to understand the benefits of a washing/sanitizing regimen using VF447 or comparable formulations would be highly desirable. VF447 may also be appropriate for reducing the risk of viral infections in areas with light soiling, such as the intermediate spaces of restaurants (e.g., around cash registers, order pick-up areas, drive-through windows, or sinks receiving minimal or restricted use). Beyond its use by the food industry, VF447 could be a good infection control tool for the cruise line and the hospitality industries, where norovirus outbreaks often occur and patrons do not always have ready access to sinks nor the discipline to perform hand washing.

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Exhibit I

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Guideline for Hand Hygiene in Health-Care Settings

Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force

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Guideline for Hand Hygiene in Health-Care Settings

Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force

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Summary

The Guideline for Hand Hygiene in Health-Care Settings provides health-care workers (HCWs) with a review of data regarding handwashing and hand antisepsis in health-care settings. In addition, it provides specific recommendations to promote improved hand-hygiene practices and reduce transmission of pathogenic microorganisms to patients and personnel in health-care settings. This report reviews studies published since the 1985 CDC guideline (Garner JS, Favero MS. CDC guideline for handwashing and hospital environmental control, 1985. Infect Control 1986;7:231–43) *and the 1995 APIC guideline* (Larson EL, APIC Guidelines Committee. APIC guideline for handwashing and hand antisepsis in health care settings. Am J Infect Control 1995;23:251–69) *were issued and provides an in-depth review of hand-hygiene practices of HCWs, levels of adherence of personnel to recommended handwashing practices, and factors adversely affecting adherence. New studies of the in vivo efficacy of alcohol-based hand rubs and the low incidence of dermatitis associated with their use are reviewed. Recent studies demonstrating the value of multidisciplinary hand-hygiene promotion programs and the potential role of alcohol-based hand rubs in improving hand-hygiene practices are summarized. Recommendations concerning related issues (e.g., the use of surgical hand antiseptics, hand lotions or creams, and wearing of artificial fingernails) are also included.*

Part I. Review of the Scientific Data Regarding Hand Hygiene

Historical Perspective

For generations, handwashing with soap and water has been considered a measure of personal hygiene (*1*). The concept of cleansing hands with an antiseptic agent probably emerged in the early 19th century. As early as 1822, a French pharmacist demonstrated that solutions containing chlorides of lime or soda could eradicate the foul odors associated with human corpses and that such solutions could be used as disinfectants and antiseptics (*2*). In a paper published in 1825, this pharmacist stated that physicians and other persons attending patients with contagious diseases would benefit from moistening their hands with a liquid chloride solution (*2*).

In 1846, Ignaz Semmelweis observed that women whose babies were delivered by students and physicians in the First Clinic at the General Hospital of Vienna consistently had a higher mortality rate than those whose babies were delivered by midwives in the Second Clinic (*3*). He noted that physicians who went directly from the autopsy suite to the obstetrics ward had a disagreeable odor on their hands despite washing their hands with soap and water upon entering the obstetrics clinic. He postulated that the puerperal fever that affected so many parturient women was caused by "cadaverous particles" transmitted from the autopsy suite to the obstetrics ward via the hands of students and physicians. Perhaps because of the known deodorizing effect of chlorine compounds, as of May 1847, he insisted that students and physicians clean their hands with a chlorine solution between each patient in the clinic. The maternal mortality rate in the First Clinic subsequently dropped dramatically and remained low for years. This intervention by Semmelweis represents the first evidence indicating that cleansing heavily contaminated hands with an antiseptic agent between patient contacts may reduce health-care–associated transmission of contagious diseases more effectively than handwashing with plain soap and water.

In 1843, Oliver Wendell Holmes concluded independently that puerperal fever was spread by the hands of health personnel (*1*). Although he described measures that could be taken to limit its spread, his recommendations had little impact on

The material in this report originated in the National Center for Infectious Diseases, James M. Hughes, M.D., Director; and the Division of Healthcare Quality Promotion, Steve Solomon, M.D., Acting Director.

obstetric practices at the time. However, as a result of the seminal studies by Semmelweis and Holmes, handwashing gradually became accepted as one of the most important measures for preventing transmission of pathogens in health-care facilities.

In 1961, the U. S. Public Health Service produced a training film that demonstrated handwashing techniques recommended for use by health-care workers (HCWs) (*4*). At the time, recommendations directed that personnel wash their hands with soap and water for 1–2 minutes before and after patient contact. Rinsing hands with an antiseptic agent was believed to be less effective than handwashing and was recommended only in emergencies or in areas where sinks were unavailable.

In 1975 and 1985, formal written guidelines on handwashing practices in hospitals were published by CDC (*5,6*). These guidelines recommended handwashing with nonantimicrobial soap between the majority of patient contacts and washing with antimicrobial soap before and after performing invasive procedures or caring for patients at high risk. Use of waterless antiseptic agents (e.g., alcohol-based solutions) was recommended only in situations where sinks were not available.

In 1988 and 1995, guidelines for handwashing and hand antisepsis were published by the Association for Professionals in Infection Control (APIC) (*7,8*). Recommended indications for handwashing were similar to those listed in the CDC guidelines. The 1995 APIC guideline included more detailed discussion of alcohol-based hand rubs and supported their use in more clinical settings than had been recommended in earlier guidelines. In 1995 and 1996, the Healthcare Infection Control Practices Advisory Committee (HICPAC) recommended that either antimicrobial soap or a waterless antiseptic agent be used for cleaning hands upon leaving the rooms of patients with multidrug-resistant pathogens (e.g., vancomycin-resistant enterococci [VRE] and methicillin-resistant *Staphylococcus aureus* [MRSA]) (*9,10*). These guidelines also provided recommendations for handwashing and hand antisepsis in other clinical settings, including routine patient care. Although the APIC and HICPAC guidelines have been adopted by the majority of hospitals, adherence of HCWs to recommended handwashing practices has remained low (*11,12*).

Recent developments in the field have stimulated a review of the scientific data regarding hand hygiene and the development of new guidelines designed to improve hand-hygiene practices in health-care facilities. This literature review and accompanying recommendations have been prepared by a Hand Hygiene Task Force, comprising representatives from HICPAC, the Society for Healthcare Epidemiology of America (SHEA), APIC, and the Infectious Diseases Society of America (IDSA).

Normal Bacterial Skin Flora

To understand the objectives of different approaches to hand cleansing, a knowledge of normal bacterial skin flora is essential. Normal human skin is colonized with bacteria; different areas of the body have varied total aerobic bacterial counts (e.g., 1×10^6 colony forming units (CFUs)/cm^2 on the scalp, 5×10^5 CFUs/cm^2 in the axilla, 4×10^4 CFUs/cm^2 on the abdomen, and 1×10^4 CFUs/cm^2 on the forearm) (*13*). Total bacterial counts on the hands of medical personnel have ranged from 3.9×10^4 to 4.6×10^6 (*14–17*). In 1938, bacteria recovered from the hands were divided into two categories: transient and resident (*14*). Transient flora, which colonize the superficial layers of the skin, are more amenable to removal by routine handwashing. They are often acquired by HCWs during direct contact with patients or contact with contaminated environmental surfaces within close proximity of the patient. Transient flora are the organisms most frequently associated with health-care–associated infections. Resident flora, which are attached to deeper layers of the skin, are more resistant to removal. In addition, resident flora (e.g., coagulase-negative staphylococci and diphtheroids) are less likely to be associated with such infections. The hands of HCWs may become persistently colonized with pathogenic flora (e.g., *S. aureus*), gram-negative bacilli, or yeast. Investigators have documented that, although the number of transient and resident flora varies considerably from person to person, it is often relatively constant for any specific person (*14,18*).

Physiology of Normal Skin

The primary function of the skin is to reduce water loss, provide protection against abrasive action and microorganisms, and act as a permeability barrier to the environment. The basic structure of skin includes, from outer- to innermost layer, the superficial region (i.e., the stratum corneum or horny layer, which is 10- to 20-µm thick), the viable epidermis (50- to 100-µm thick), the dermis (1- to 2-mm thick), and the hypodermis (1- to 2-mm thick). The barrier to percutaneous absorption lies within the stratum corneum, the thinnest and smallest compartment of the skin. The stratum corneum contains the corneocytes (or horny cells), which are flat, polyhedral-shaped nonnucleated cells, remnants of the terminally differentiated keratinocytes located in the viable epidermis. Corneocytes are composed primarily of insoluble bundled keratins surrounded by a cell envelope stabilized by cross-linked proteins and covalently bound lipid. Interconnecting the corneocytes of the stratum corneum are polar structures (e.g., corneodesmosomes), which contribute to stratum corneum cohesion.

The intercellular region of the stratum corneum is composed of lipid primarily generated from the exocytosis of lamellar bodies during the terminal differentiation of the keratinocytes. The intercellular lipid is required for a competent skin barrier and forms the only continuous domain. Directly under the stratum corneum is a stratified epidermis, which is composed primarily of 10–20 layers of keratinizing epithelial cells that are responsible for the synthesis of the stratum corneum. This layer also contains melanocytes involved in skin pigmentation; Langerhans cells, which are important for antigen presentation and immune responses; and Merkel cells, whose precise role in sensory reception has yet to be fully delineated. As keratinocytes undergo terminal differentiation, they begin to flatten out and assume the dimensions characteristic of the corneocytes (i.e., their diameter changes from 10–12 μm to 20–30 μm, and their volume increases by 10- to 20-fold). The viable epidermis does not contain a vascular network, and the keratinocytes obtain their nutrients from below by passive diffusion through the interstitial fluid.

The skin is a dynamic structure. Barrier function does not simply arise from the dying, degeneration, and compaction of the underlying epidermis. Rather, the processes of cornification and desquamation are intimately linked; synthesis of the stratum corneum occurs at the same rate as loss. Substantial evidence now confirms that the formation of the skin barrier is under homeostatic control, which is illustrated by the epidermal response to barrier perturbation by skin stripping or solvent extraction. Circumstantial evidence indicates that the rate of keratinocyte proliferation directly influences the integrity of the skin barrier. A general increase in the rate of proliferation results in a decrease in the time available for 1) uptake of nutrients (e.g., essential fatty acids), 2) protein and lipid synthesis, and 3) processing of the precursor molecules required for skin-barrier function. Whether chronic but quantitatively smaller increases in rate of epidermal proliferation also lead to changes in skin-barrier function remains unclear. Thus, the extent to which the decreased barrier function caused by irritants is caused by an increased epidermal proliferation also is unknown.

The current understanding of the formation of the stratum corneum has come from studies of the epidermal responses to perturbation of the skin barrier. Experimental manipulations that disrupt the skin barrier include 1) extraction of skin lipids with apolar solvents, 2) physical stripping of the stratum corneum using adhesive tape, and 3) chemically induced irritation. All of these experimental manipulations lead to a decreased skin barrier as determined by transepidermal water loss (TEWL). The most studied experimental system is the treatment of mouse skin with acetone. This experiment results in a marked and immediate increase in TEWL, and therefore a decrease in skin-barrier function. Acetone treatment selectively removes glycerolipids and sterols from the skin, which indicates that these lipids are necessary, though perhaps not sufficient in themselves, for barrier function. Detergents act like acetone on the intercellular lipid domain. The return to normal barrier function is biphasic: 50%–60% of barrier recovery typically occurs within 6 hours, but complete normalization of barrier function requires 5–6 days.

Definition of Terms

Alcohol-based hand rub. An alcohol-containing preparation designed for application to the hands for reducing the number of viable microorganisms on the hands. In the United States, such preparations usually contain 60%–95% ethanol or isopropanol.

Antimicrobial soap. Soap (i.e., detergent) containing an antiseptic agent.

Antiseptic agent. Antimicrobial substances that are applied to the skin to reduce the number of microbial flora. Examples include alcohols, chlorhexidine, chlorine, hexachlorophene, iodine, chloroxylenol (PCMX), quaternary ammonium compounds, and triclosan.

Antiseptic handwash. Washing hands with water and soap or other detergents containing an antiseptic agent.

Antiseptic hand rub. Applying an antiseptic hand-rub product to all surfaces of the hands to reduce the number of microorganisms present.

Cumulative effect. A progressive decrease in the numbers of microorganisms recovered after repeated applications of a test material.

Decontaminate hands. To Reduce bacterial counts on hands by performing antiseptic hand rub or antiseptic handwash.

Detergent. Detergents (i.e., surfactants) are compounds that possess a cleaning action. They are composed of both hydrophilic and lipophilic parts and can be divided into four groups: anionic, cationic, amphoteric, and nonionic detergents. Although products used for handwashing or antiseptic handwash in health-care settings represent various types of detergents, the term "soap" is used to refer to such detergents in this guideline.

Hand antisepsis. Refers to either antiseptic handwash or antiseptic hand rub.

Hand hygiene. A general term that applies to either handwashing, antiseptic handwash, antiseptic hand rub, or surgical hand antisepsis.

Handwashing. Washing hands with plain (i.e., non-antimicrobial) soap and water.

Persistent activity. Persistent activity is defined as the prolonged or extended antimicrobial activity that prevents or inhibits the proliferation or survival of microorganisms after application of the product. This activity may be demonstrated by sampling a site several minutes or hours after application and demonstrating bacterial antimicrobial effectiveness when compared with a baseline level. This property also has been referred to as "residual activity." Both substantive and nonsubstantive active ingredients can show a persistent effect if they substantially lower the number of bacteria during the wash period.

Plain soap. Plain soap refers to detergents that do not contain antimicrobial agents or contain low concentrations of antimicrobial agents that are effective solely as preservatives.

Substantivity. Substantivity is an attribute of certain active ingredients that adhere to the stratum corneum (i.e., remain on the skin after rinsing or drying) to provide an inhibitory effect on the growth of bacteria remaining on the skin.

Surgical hand antisepsis. Antiseptic handwash or antiseptic hand rub performed preoperatively by surgical personnel to eliminate transient and reduce resident hand flora. Antiseptic detergent preparations often have persistent antimicrobial activity.

Visibly soiled hands. Hands showing visible dirt or visibly contaminated with proteinaceous material, blood, or other body fluids (e.g., fecal material or urine).

Waterless antiseptic agent. An antiseptic agent that does not require use of exogenous water. After applying such an agent, the hands are rubbed together until the agent has dried.

Food and Drug Administration (FDA) product categories. The 1994 FDA Tentative Final Monograph for Health-Care Antiseptic Drug Products divided products into three categories and defined them as follows (*19*):

- *Patient preoperative skin preparation.* A fast-acting, broad-spectrum, and persistent antiseptic-containing preparation that substantially reduces the number of microorganisms on intact skin.
- *Antiseptic handwash or HCW handwash.* An antiseptic-containing preparation designed for frequent use; it reduces the number of microorganisms on intact skin to an initial baseline level after adequate washing, rinsing, and drying; it is broad-spectrum, fast-acting, and if possible, persistent.
- *Surgical hand scrub.* An antiseptic-containing preparation that substantially reduces the number of microorganisms on intact skin; it is broad-spectrum, fast-acting, and persistent.

Evidence of Transmission of Pathogens on Hands

Transmission of health-care–associated pathogens from one patient to another via the hands of HCWs requires the following sequence of events:

- Organisms present on the patient's skin, or that have been shed onto inanimate objects in close proximity to the patient, must be transferred to the hands of HCWs.
- These organisms must then be capable of surviving for at least several minutes on the hands of personnel.
- Next, handwashing or hand antisepsis by the worker must be inadequate or omitted entirely, or the agent used for hand hygiene must be inappropriate.
- Finally, the contaminated hands of the caregiver must come in direct contact with another patient, or with an inanimate object that will come into direct contact with the patient.

Health-care–associated pathogens can be recovered not only from infected or draining wounds, but also from frequently colonized areas of normal, intact patient skin (*20–31*). The perineal or inguinal areas are usually most heavily colonized, but the axillae, trunk, and upper extremities (including the hands) also are frequently colonized (*23,25,26,28,30–32*). The number of organisms (e.g., *S. aureus, Proteus mirabilis, Klebsiella* spp., and *Acinetobacter* spp.) present on intact areas of the skin of certain patients can vary from 100 to $10^6/cm^2$ (*25,29,31,33*). Persons with diabetes, patients undergoing dialysis for chronic renal failure, and those with chronic dermatitis are likely to have areas of intact skin that are colonized with *S. aureus* (*34–41*). Because approximately 10^6 skin squames containing viable microorganisms are shed daily from normal skin (*42*), patient gowns, bed linen, bedside furniture, and other objects in the patient's immediate environment can easily become contaminated with patient flora (*30,43–46*). Such contamination is particularly likely to be caused by staphylococci or enterococci, which are resistant to dessication.

Data are limited regarding the types of patient-care activities that result in transmission of patient flora to the hands of personnel (*26,45–51*). In the past, attempts have been made to stratify patient-care activities into those most likely to cause hand contamination (*52*), but such stratification schemes were never validated by quantifying the level of bacterial contamination that occurred. Nurses can contaminate their hands with 100–1,000 CFUs of *Klebsiella* spp. during "clean" activities (e.g., lifting a patient; taking a patient's pulse, blood pressure, or oral temperature; or touching a patient's hand, shoulder, or groin) (*48*). Similarly, in another study, hands were cultured of nurses who touched the groins of patients heavily colonized with *P. mirabilis* (*25*); 10–600 CFUs/mL of this

organism were recovered from glove juice samples from the nurses' hands. Recently, other researchers studied contamination of HCWs' hands during activities that involved direct patient-contact wound care, intravascular catheter care, respiratory-tract care, and the handling of patient secretions (*51*). Agar fingertip impression plates were used to culture bacteria; the number of bacteria recovered from fingertips ranged from 0 to 300 CFUs. Data from this study indicated that direct patient contact and respiratory-tract care were most likely to contaminate the fingers of caregivers. Gram-negative bacilli accounted for 15% of isolates and *S. aureus* for 11%. Duration of patient-care activity was strongly associated with the intensity of bacterial contamination of HCWs' hands.

HCWs can contaminate their hands with gram-negative bacilli, *S. aureus*, enterococci, or *Clostridium difficile* by performing "clean procedures" or touching intact areas of the skin of hospitalized patients (*26,45,46,53*). Furthermore, personnel caring for infants with respiratory syncytial virus (RSV) infections have acquired RSV by performing certain activities (e.g., feeding infants, changing diapers, and playing with infants) (*49*). Personnel who had contact only with surfaces contaminated with the infants' secretions also acquired RSV by contaminating their hands with RSV and inoculating their oral or conjunctival mucosa. Other studies also have documented that HCWs may contaminate their hands (or gloves) merely by touching inanimate objects in patient rooms (*46,53–56*). None of the studies concerning hand contamination of hospital personnel were designed to determine if the contamination resulted in transmission of pathogens to susceptible patients.

Other studies have documented contamination of HCWs' hands with potential health-care–associated pathogens, but did not relate their findings to the specific type of preceding patient contact (*15,17,57–62*). For example, before glove use was common among HCWs, 15% of nurses working in an isolation unit carried a median of 1 x 10^4 CFUs of *S. aureus* on their hands (*61*). Of nurses working in a general hospital, 29% had *S. aureus* on their hands (median count: 3,800 CFUs), whereas 78% of those working in a hospital for dermatology patients had the organism on their hands (median count: 14.3 x 10^6 CFUs). Similarly, 17%–30% of nurses carried gram-negative bacilli on their hands (median counts: 3,400–38,000 CFUs). One study found that *S. aureus* could be recovered from the hands of 21% of intensive-care–unit personnel and that 21% of physician and 5% of nurse carriers had >1,000 CFUs of the organism on their hands (*59*). Another study found lower levels of colonization on the hands of personnel working in a neurosurgery unit, with an average of 3 CFUs of *S. aureus* and 11 CFUs of gram-negative bacilli (*16*). Serial cultures revealed that 100% of HCWs carried gram-negative bacilli at least once, and 64% carried *S. aureus* at least once.

Models of Hand Transmission

Several investigators have studied transmission of infectious agents by using different experimental models. In one study, nurses were asked to touch the groins of patients heavily colonized with gram-negative bacilli for 15 seconds — as though they were taking a femoral pulse (*25*). Nurses then cleaned their hands by washing with plain soap and water or by using an alcohol hand rinse. After cleaning their hands, they touched a piece of urinary catheter material with their fingers, and the catheter segment was cultured. The study revealed that touching intact areas of moist skin of the patient transferred enough organisms to the nurses' hands to result in subsequent transmission to catheter material, despite handwashing with plain soap and water.

The transmission of organisms from artificially contaminated "donor" fabrics to clean "recipient" fabrics via hand contact also has been studied. Results indicated that the number of organisms transmitted was greater if the donor fabric or the hands were wet upon contact (*63*). Overall, only 0.06% of the organisms obtained from the contaminated donor fabric were transferred to recipient fabric via hand contact. *Staphylococcus saprophyticus, Pseudomonas aeruginosa,* and *Serratia* spp. were also transferred in greater numbers than was *Escherichia coli* from contaminated fabric to clean fabric after hand contact (*64*). Organisms are transferred to various types of surfaces in much larger numbers (i.e., >10^4) from wet hands than from hands that are thoroughly dried (*65*).

Relation of Hand Hygiene and Acquisition of Health-Care–Associated Pathogens

Hand antisepsis reduces the incidence of health-care–associated infections (*66,67*). An intervention trial using historical controls demonstrated in 1847 that the mortality rate among mothers who delivered in the First Obstetrics Clinic at the General Hospital of Vienna was substantially lower when hospital staff cleaned their hands with an antiseptic agent than when they washed their hands with plain soap and water (*3*).

In the 1960s, a prospective, controlled trial sponsored by the National Institutes of Health and the Office of the Surgeon General demonstrated that infants cared for by nurses who did not wash their hands after handling an index infant colonized with *S. aureus* acquired the organism more often and more rapidly than did infants cared for by nurses who used hexachlorophene to clean their hands between infant

contacts (*68*). This trial provided evidence that, when compared with no handwashing, washing hands with an antiseptic agent between patient contacts reduces transmission of health-care–associated pathogens.

Trials have studied the effects of handwashing with plain soap and water versus some form of hand antisepsis on health-care–associated infection rates (*69,70*). Health-care–associated infection rates were lower when antiseptic handwashing was performed by personnel (*69*). In another study, antiseptic handwashing was associated with lower health-care–associated infection rates in certain intensive-care units, but not in others (70).

Health-care–associated infection rates were lower after antiseptic handwashing using a chlorhexidine-containing detergent compared with handwashing with plain soap or use of an alcohol-based hand rinse (*71*). However, because only a minimal amount of the alcohol rinse was used during periods when the combination regimen also was in use and because adherence to policies was higher when chlorhexidine was available, determining which factor (i.e., the hand-hygiene regimen or differences in adherence) accounted for the lower infection rates was difficult. Investigators have determined also that health-care–associated acquisition of MRSA was reduced when the antimicrobial soap used for hygienic handwashing was changed (*72,73*).

Increased handwashing frequency among hospital staff has been associated with decreased transmission of *Klebsiella* spp. among patients (*48*); these studies, however, did not quantitate the level of handwashing among personnel. In a recent study, the acquisition of various health-care–associated pathogens was reduced when hand antisepsis was performed more frequently by hospital personnel (*74*); both this study and another (*75*) documented that the prevalence of health-care–associated infections decreased as adherence to recommended hand-hygiene measures improved.

Outbreak investigations have indicated an association between infections and understaffing or overcrowding; the association was consistently linked with poor adherence to hand hygiene. During an outbreak investigation of risk factors for central venous catheter-associated bloodstream infections (*76*), after adjustment for confounding factors, the patient-to-nurse ratio remained an independent risk factor for bloodstream infection, indicating that nursing staff reduction below a critical threshold may have contributed to this outbreak by jeopardizing adequate catheter care. The understaffing of nurses can facilitate the spread of MRSA in intensive-care settings (*77*) through relaxed attention to basic control measures (e.g., hand hygiene). In an outbreak of *Enterobacter cloacae* in a neonatal intensive-care unit (*78*), the daily number of hospitalized children was above the maximum capacity of the unit, resulting in an available space per child below current recommendations. In parallel, the number of staff members on duty was substantially less than the number necessitated by the workload, which also resulted in relaxed attention to basic infection-control measures. Adherence to hand-hygiene practices before device contact was only 25% during the workload peak, but increased to 70% after the end of the understaffing and overcrowding period. Surveillance documented that being hospitalized during this period was associated with a fourfold increased risk of acquiring a health-care–associated infection. This study not only demonstrates the association between workload and infections, but it also highlights the intermediate cause of antimicrobial spread: poor adherence to hand-hygiene policies.

Methods Used To Evaluate the Efficacy of Hand-Hygiene Products

Current Methods

Investigators use different methods to study the in vivo efficacy of handwashing, antiseptic handwash, and surgical hand antisepsis protocols. Differences among the various studies include 1) whether hands are purposely contaminated with bacteria before use of test agents, 2) the method used to contaminate fingers or hands, 3) the volume of hand-hygiene product applied to the hands, 4) the time the product is in contact with the skin, 5) the method used to recover bacteria from the skin after the test solution has been used, and 6) the method of expressing the efficacy of the product (i.e., either percent reduction in bacteria recovered from the skin or log reduction of bacteria released from the skin). Despite these differences, the majority of studies can be placed into one of two major categories: studies focusing on products to remove transient flora and studies involving products that are used to remove resident flora from the hands. The majority of studies of products for removing transient flora from the hands of HCWs involve artificial contamination of the volunteer's skin with a defined inoculum of a test organism before the volunteer uses a plain soap, an antimicrobial soap, or a waterless antiseptic agent. In contrast, products tested for the preoperative cleansing of surgeons' hands (which must comply with surgical hand-antisepsis protocols) are tested for their ability to remove resident flora from without artificially contaminating the volunteers' hands.

In the United States, antiseptic handwash products intended for use by HCWs are regulated by FDA's Division of Over-the-Counter Drug Products (OTC). Requirements for in vitro and in vivo testing of HCW handwash products and surgical

hand scrubs are outlined in the FDA Tentative Final Monograph for Healthcare Antiseptic Drug Products (TFM) (*19*). Products intended for use as HCW handwashes are evaluated by using a standardized method (*19*). Tests are performed in accordance with use directions for the test material. Before baseline bacterial sampling and before each wash with the test material, 5 mL of a standardized suspension of *Serratia marcescens* are applied to the hands and then rubbed over the surfaces of the hands. A specified volume of the test material is dispensed into the hands and is spread over the hands and lower one third of the forearms. A small amount of tap water is added to the hands, and hands are completely lathered for a specified time, covering all surfaces of the hands and the lower third of the forearms. Volunteers then rinse hands and forearms under 40°C tap water for 30 seconds. Ten washes with the test formulation are required. After the first, third, seventh, and tenth washes, rubber gloves or polyethylene bags used for sampling are placed on the right and left hands, and 75 mL of sampling solution is added to each glove; gloves are secured above the wrist. All surfaces of the hand are massaged for 1 minute, and samples are obtained aseptically for quantitative culture. No neutralizer of the antimicrobial is routinely added to the sampling solution, but if dilution of the antimicrobial in the sampling fluid does not result in demonstrable neutralization, a neutralizer specific for the test formulation is added to the sampling solution. For waterless formulations, a similar procedure is used. TFM criteria for efficacy are as follows: a 2-$\log_{10}$ reduction of the indicator organism on each hand within 5 minutes after the first use, and a 3-$\log_{10}$ reduction of the indicator organism on each hand within 5 minutes after the tenth use (*19*).

Products intended for use as surgical hand scrubs have been evaluated also by using a standardized method (*19*). Volunteers clean under fingernails with a nail stick and clip their fingernails. All jewelry is removed from hands and arms. Hands and two thirds of forearms are rinsed with tap water (38°C–42°C) for 30 seconds, and then they are washed with a nonantimicrobial soap for 30 seconds and are rinsed for 30 seconds under tap water. Baseline microbial hand counts can then be determined. Next, a surgical scrub is performed with the test formulation using directions provided by the manufacturer. If no instructions are provided with the formulation, two 5-minute scrubs of hands and forearms followed by rinsing are performed. Reduction from baseline microbial hand counts is determined in a series of 11 scrubs conducted during 5 days. Hands are sampled at 1 minute, 3 hours, and 6 hours after the first scrubs on day 1, day 2, and day 5. After washing, volunteers wear rubber gloves; 75 mL of sampling solution are then added to one glove, and all surfaces of the hands are massaged for 1 minute. Samples are then taken aseptically and cultured quantitatively. The other glove remains on the other hand for 6 hours and is sampled in the same manner. TFM requires that formulations reduce the number of bacteria 1 $\log_{10}$ on each hand within 1 minute of product application and that the bacterial cell count on each hand does not subsequently exceed baseline within 6 hours on day 1; the formulation must produce a 2-$\log_{10}$ reduction in microbial flora on each hand within 1 minute of product application by the end of the second day of enumeration and a 3-$\log_{10}$ reduction of microbial flora on each hand within 1 minute of product use by the end of the fifth day when compared with the established baseline (*19*).

The method most widely used in Europe to evaluate the efficacy of hand-hygiene agents is European Standard 1500–1997 (EN 1500—Chemical disinfectants and antiseptics. Hygienic hand-rub test method and requirements) (*79*). This method requires 12–15 test volunteers and an 18- to 24-hour growth of broth culture of *E. coli* K12. Hands are washed with a soft soap, dried, and then immersed halfway to the metacarpals in the broth culture for 5 seconds. Hands are removed from the broth culture, excess fluid is drained off, and hands are dried in the air for 3 minutes. Bacterial recovery for the initial value is obtained by kneading the fingertips of each hand separately for 60 seconds in 10 mL of tryptic soy broth (TSB) without neutralizers. The hands are removed from the broth and disinfected with 3 mL of the hand-rub agent for 30 seconds in a set design. The same operation is repeated with total disinfection time not exceeding 60 seconds. Both hands are rinsed in running water for 5 seconds and water is drained off. Fingertips of each hand are kneaded separately in 10 mL of TSB with added neutralizers. These broths are used to obtain the final value. Log_{10} dilutions of recovery medium are prepared and plated out. Within 3 hours, the same volunteers are tested with the reference disinfectant (60% 2-propanol [isopropanol]) and the test product. Colony counts are performed after 24 and 48 hours of incubation at 36°C. The average colony count of both left and right hand is used for evaluation. The log-reduction factor is calculated and compared with the initial and final values. The reduction factor of the test product should be superior or the same as the reference alcohol-based rub for acceptance. If a difference exists, then the results are analyzed statistically using the Wilcoxon test. Products that have log reductions substantially less than that observed with the reference alcohol-based hand rub (i.e., approximately 4 $\log_{10}$ reduction) are classified as not meeting the standard.

Because of different standards for efficacy, criteria cited in FDA TFM and the European EN 1500 document for establishing alcohol-based hand rubs vary (*1,19,79*). Alcohol-based

hand rubs that meet TFM criteria for efficacy may not necessarily meet the EN 1500 criteria for efficacy (*80*). In addition, scientific studies have not established the extent to which counts of bacteria or other microorganisms on the hands need to be reduced to minimize transmission of pathogens in health-care facilities (*1,8*); whether bacterial counts on the hands must be reduced by 1 $\log_{10}$ (90% reduction), 2 $\log_{10}$ (99%), 3 $\log_{10}$ (99.9%), or 4 $\log_{10}$ (99.99%) is unknown. Several other methods also have been used to measure the efficacy of antiseptic agents against various viral pathogens (*81–83*).

Shortcomings of Traditional Methodologies

Accepted methods of evaluating hand-hygiene products intended for use by HCWs require that test volunteers wash their hands with a plain or antimicrobial soap for 30 seconds or 1 minute, despite the observation in the majority of studies that the average duration of handwashing by hospital personnel is <15 seconds (*52,84–89*). A limited number of investigators have used 15-second handwashing or hygienic hand-wash protocols (*90–94*). Therefore, almost no data exist regarding the efficacy of plain or antimicrobial soaps under conditions in which they are actually used by HCWs. Similarly, certain accepted methods for evaluating waterless antiseptic agents for use as antiseptic hand rubs require that 3 mL of alcohol be rubbed into the hands for 30 seconds, followed by a repeat application for the same duration. This type of protocol also does not reflect actual usage patterns among HCWs. Furthermore, volunteers used in evaluations of products are usually surrogates for HCWs, and their hand flora may not reflect flora found on the hands of personnel working in health-care settings. Further studies should be conducted among practicing HCWs using standardized protocols to obtain more realistic views of microbial colonization and risk of bacterial transfer and cross-transmission (*51*).

Review of Preparations Used for Hand Hygiene

Plain (Non-Antimicrobial) Soap

Soaps are detergent-based products that contain esterified fatty acids and sodium or potassium hydroxide. They are available in various forms including bar soap, tissue, leaflet, and liquid preparations. Their cleaning activity can be attributed to their detergent properties, which result in removal of dirt, soil, and various organic substances from the hands. Plain soaps have minimal, if any, antimicrobial activity. However, handwashing with plain soap can remove loosely adherent transient flora. For example, handwashing with plain soap and water for 15 seconds reduces bacterial counts on the skin by 0.6–1.1 $\log_{10}$, whereas washing for 30 seconds reduces counts by 1.8–2.8 $\log_{10}$ (*1*). However, in several studies, handwashing with plain soap failed to remove pathogens from the hands of hospital personnel (*25,45*). Handwashing with plain soap can result in paradoxical increases in bacterial counts on the skin (*92,95–97*). Non-antimicrobial soaps may be associated with considerable skin irritation and dryness (*92,96,98*), although adding emollients to soap preparations may reduce their propensity to cause irritation. Occasionally, plain soaps have become contaminated, which may lead to colonization of hands of personnel with gram-negative bacilli (*99*).

Alcohols

The majority of alcohol-based hand antiseptics contain either isopropanol, ethanol, n-propanol, or a combination of two of these products. Although n-propanol has been used in alcohol-based hand rubs in parts of Europe for many years, it is not listed in TFM as an approved active agent for HCW handwashes or surgical hand-scrub preparations in the United States. The majority of studies of alcohols have evaluated individual alcohols in varying concentrations. Other studies have focused on combinations of two alcohols or alcohol solutions containing limited amounts of hexachlorophene, quaternary ammonium compounds, povidone-iodine, triclosan, or chlorhexidine gluconate (*61,93,100–119*).

The antimicrobial activity of alcohols can be attributed to their ability to denature proteins (*120*). Alcohol solutions containing 60%–95% alcohol are most effective, and higher concentrations are less potent (*120–122*) because proteins are not denatured easily in the absence of water (*120*). The alcohol content of solutions may be expressed as percent by weight (w/w), which is not affected by temperature or other variables, or as percent by volume (vol/vol), which can be affected by temperature, specific gravity, and reaction concentration (*123*). For example, 70% alcohol by weight is equivalent to 76.8% by volume if prepared at 15°C, or 80.5% if prepared at 25°C (*123*). Alcohol concentrations in antiseptic hand rubs are often expressed as percent by volume (*19*).

Alcohols have excellent in vitro germicidal activity against gram-positive and gram-negative vegetative bacteria, including multidrug-resistant pathogens (e.g., MRSA and VRE), *Mycobacterium tuberculosis*, and various fungi (*120–122,124–129*). Certain enveloped (lipophilic) viruses (e.g., herpes simplex virus, human immunodeficiency virus [HIV], influenza virus, respiratory syncytial virus, and vaccinia virus) are susceptible to alcohols when tested in vitro (*120,130,131*) (Table 1). Hepatitis B virus is an enveloped virus that is somewhat less susceptible but is killed by 60%–70% alcohol; hepatitis C virus also is likely killed by this percentage of alcohol (*132*). In a porcine tissue carrier model used to study antiseptic activity, 70% ethanol and 70% isopropanol were found to

TABLE 1. Virucidal activity of antiseptic agents against enveloped viruses

Ref. no.	Test method	Viruses	Agent	Results
(*379*)	Suspension	HIV	19% EA	LR = 2.0 in 5 minutes
(*380*)	Suspension	HIV	50% EA 35% IPA	LR > 3.5 LR > 3.7
(*381*)	Suspension	HIV	70% EA	LR = 7.0 in 1 minute
(*382*)	Suspension	HIV	70% EA	LR = 3.2B 5.5 in 30 seconds
(*383*)	Suspension	HIV	70% IPA/0.5% CHG 4% CHG	LR = 6.0 in 15 seconds LR = 6.0 in 15 seconds
(*384*)	Suspension	HIV	Chloroxylenol Benzalkonium chloride	Inactivated in 1 minute Inactivated in 1 minute
(*385*)	Suspension	HIV	Povidone-iodine Chlorhexidine	Inactivated Inactivated
(*386*)	Suspension	HIV	Detergent/0.5% PCMX	Inactivated in 30 seconds
(*387*)	Suspension/dried plasma chimpanzee challenge	HBV	70% IPA	LR = 6.0 in 10 minutes
(*388*)	Suspension/plasma chimpanzee challenge	HBV	80% EA	LR = 7.0 in 2 minutes
(*389*)	Suspension	HSV	95% EA 75% EA 95% IPA 70% EA + 0.5% CHG	LR > 5.0 in 1 minute LR > 5.0 LR > 5.0 LR > 5.0
(*130*)	Suspension	RSV	35% IPA 4% CHG	LR > 4.3 in 1 minute LR > 3.3
(*141*)	Suspension	Influenza Vaccinia	95% EA 95% EA	Undetectable in 30 seconds Undetectable in 30 seconds
(*141*)	Hand test	Influenza Vaccinia	95% EA 95% EA	LR > 2.5 LR > 2.5

Note: HIV = human immunodeficiency virus, EA = ethanol, LR = Log_{10} reduction, IPA = isopropanol, CHG = chlorhexidine gluconate, HBV = hepatitis B virus, RSV = respiratory syncitial virus, HSV = herpes simplex virus, HAV = hepatitis A virus, and PCMX = chloroxylenol.

reduce titers of an enveloped bacteriophage more effectively than an antimicrobial soap containing 4% chlorhexidine gluconate (*133*). Despite its effectiveness against these organisms, alcohols have very poor activity against bacterial spores, protozoan oocysts, and certain nonenveloped (nonlipophilic) viruses.

Numerous studies have documented the *in vivo* antimicrobial activity of alcohols. Alcohols effectively reduce bacterial counts on the hands (*14,121,125,134*). Typically, log reductions of the release of test bacteria from artificially contaminated hands average 3.5 log_{10} after a 30-second application and 4.0–5.0 log_{10} after a 1-minute application (*1*). In 1994, the FDA TFM classified ethanol 60%–95% as a Category I agent (i.e., generally safe and effective for use in antiseptic handwash or HCW hand-wash products) (*19*). Although TFM placed isopropanol 70%–91.3% in category IIIE (i.e., insufficient data to classify as effective), 60% isopropanol has subsequently been adopted in Europe as the reference standard against which alcohol-based hand-rub products are compared (*79*). Alcohols are rapidly germicidal when applied to the skin, but they have no appreciable persistent (i.e., residual) activity. However, regrowth of bacteria on the skin occurs slowly after use of alcohol-based hand antiseptics, presumably because of the sublethal effect alcohols have on some of the skin bacteria (*135,136*). Addition of chlorhexidine, quaternary ammonium compounds, octenidine, or triclosan to alcohol-based solutions can result in persistent activity (*1*).

Alcohols, when used in concentrations present in alcohol-based hand rubs, also have in vivo activity against several nonenveloped viruses (Table 2). For example, 70% isopropanol and 70% ethanol are more effective than medicated soap or nonmedicated soap in reducing rotavirus titers on fingerpads (*137,138*). A more recent study using the same test methods evaluated a commercially available product containing 60%

TABLE 2. Virucidal activity of antiseptic agents against nonenveloped viruses

Ref. no.	Test method	Viruses	Antiseptic	Result
(*390*)	Suspension	Rotavirus	4% CHG	LR < 3.0 in 1 minute
			10% Povidone-Iodine	LR > 3.0
			70% IPA/0.1% HCP	LR > 3.0
(*141*)	Hand test	Adenovirus	95% EA	LR > 1.4
		Poliovirus	95% EA	LR = 0.2–1.0
		Coxsackie	95% EA	LR = 1.1–1.3
	Finger test	Adenovirus	95% EA	LR > 2.3
		Poliovirus	95% EA	LR = 0.7–2.5
		Coxsackie	95% EA	LR = 2.9
(*389*)	Suspension	ECHO virus	95% EA	LR > 3.0 in 1 minute
			75% EA	LR ≤ 1.0
			95% IPA	LR = 0
			70% IPA + 0.5% CHG	LR = 0
(*140*)	Finger pad	HAV	70% EA	87.4% reduction
			62% EA foam	89.3% reduction
			plain soap	78.0% reduction
			4% CHG	89.6% reduction
			0.3% Triclosan	92.0% reduction
(*105*)	Finger tips	Bovine Rotavirus	n-propanol + IPA	LR = 3.8 in 30 seconds
			70% IPA	LR = 3.1
			70% EA	LR = 2.9
			2% triclosan	LR = 2.1
			water (control)	LR = 1.3
			7.5% povidone-iodine	LR = 1.3
			plain soap	LR = 1.2
			4% CHG	LR = 0.5
(*137*)	Finger pad	Human Rotavirus	70% IPA	98.9% decrease in 10 seconds
			plain soap	77.1%
(*138*)	Finger pad	Human Rotavirus	70% IPA	99.6% decrease in 10 seconds
			2% CHG	80.3%
			plain soap	72.5%
(*81*)	Finger pad	Rotavirus	60% EA gel	LR > 3.0 in 10 seconds
		Rhinovirus	60% EA gel	LR > 3.0
		Adenovirus	60% EA gel	LR > 3.0
(*139*)	Finger pad	Poliovirus	70% EA	LR = 1.6 in 10 seconds
			70% IPA	LR = 0.8
(*200*)	Finger tips	Poliovirus	Plain soap	LR = 2.1
			80% EA	LR = 0.4

Note: HIV = human immunodeficiency virus, EA = ethanol, LR = Log_{10} reduction, IPA = isopropanol, CHG = chlorhexidine gluconate, HBV = hepatitis B virus, RSV = respiratory syncitial virus, HSV = herpes simplex virus, and HAV = hepatitis A virus.

ethanol and found that the product reduced the infectivity titers of three nonenveloped viruses (i.e., rotavirus, adenovirus, and rhinovirus) by >3 logs (*81*). Other nonenveloped viruses such as hepatitis A and enteroviruses (e.g., poliovirus) may require 70%–80% alcohol to be reliably inactivated (*82,139*). However, both 70% ethanol and a 62% ethanol foam product with emollients reduced hepatitis A virus titers on whole hands or fingertips more than nonmedicated soap; both were equally as effective as antimicrobial soap containing 4% chlorhexidine gluconate in reducing reduced viral counts on hands (*140*). In the same study, both 70% ethanol and the 62% ethanol foam product demonstrated greater virucidal activity against poliovirus than either non-antimicrobial soap or a 4% chlorhexidine gluconate-containing soap (*140*). However, depending on the alcohol concentration, the amount of time that hands are exposed to the alcohol, and viral variant, alcohol may not be effective against hepatitis A and other nonlipophilic viruses. The inactivation of nonenveloped viruses is influenced by temperature, disinfectant-virus volume ratio, and protein load (*141*). Ethanol has greater activity against viruses than isopropanol. Further in vitro and in vivo studies of both alcohol-based formulations and antimicrobial soaps are warranted to establish the minimal level of virucidal activity that is required to interrupt direct contact transmission of viruses in health-care settings.

Alcohols are not appropriate for use when hands are visibly dirty or contaminated with proteinaceous materials. However, when relatively small amounts of proteinaceous material (e.g., blood) are present, ethanol and isopropanol may reduce viable bacterial counts on hands more than plain soap or antimicrobial soap (*142*).

Alcohol can prevent the transfer of health-care–associated pathogens (*25,63,64*). In one study, gram-negative bacilli were transferred from a colonized patient's skin to a piece of catheter material via the hands of nurses in only 17% of experiments after antiseptic hand rub with an alcohol-based hand rinse (*25*). In contrast, transfer of the organisms occurred in 92% of experiments after handwashing with plain soap and water. This experimental model indicates that when the hands of HCWs are heavily contaminated, an antiseptic hand rub using an alcohol-based rinse can prevent pathogen transmission more effectively than can handwashing with plain soap and water.

Alcohol-based products are more effective for standard handwashing or hand antisepsis by HCWs than soap or antimicrobial soaps (Table 3) (*25,53,61,93,106–112,119,143–152*). In all but two of the trials that compared alcohol-based solutions with antimicrobial soaps or detergents, alcohol reduced bacterial counts on hands more than washing hands with soaps or detergents containing hexachlorophene, povidone-iodine, 4% chlorhexidine, or triclosan. In studies examining antimicrobial-resistant organisms, alcohol-based products reduced the number of multidrug-resistant pathogens recovered from the hands of HCWs more effectively than did handwashing with soap and water (*153–155*).

Alcohols are effective for preoperative cleaning of the hands of surgical personnel (*1,101,104,113–119,135,143,147,156–159*) (Tables 4 and 5). In multiple studies, bacterial counts on the hands were determined immediately after using the product and again 1–3 hours later; the delayed testing was performed to determine if regrowth of bacteria on the hands is inhibited during operative procedures. Alcohol-based solutions were more effective than washing hands with plain soap in all studies, and they reduced bacterial counts on the hands more than antimicrobial soaps or detergents in the majority of experiments (*101,104,113–119,135,143,147,157–159*). In addition, the majority of alcohol-based preparations were more effective than povidone-iodine or chlorhexidine.

The efficacy of alcohol-based hand-hygiene products is affected by several factors, including the type of alcohol used, concentration of alcohol, contact time, volume of alcohol used, and whether the hands are wet when the alcohol is applied. Applying small volumes (i.e., 0.2–0.5 mL) of alcohol to the hands is not more effective than washing hands with plain soap and water (*63,64*). One study documented that 1 mL of alcohol was substantially less effective than 3 mL (*91*). The ideal volume of product to apply to the hands is not known

TABLE 3. Studies comparing the relative efficacy (based on log_{10} reductions achieved) of plain soap or antimicrobial soaps versus alcohol-based antiseptics in reducing counts of viable bacteria on hands

Ref. no.	Year	Skin contamination	Assay method	Time (sec)	Relative efficacy
(*143*)	1965	Existing hand flora	Finger-tip agar culture	60	Plain soap < HCP < 50% EA foam
(*119*)	1975	Existing hand flora	Hand-rub broth culture	—	Plain soap < 95% EA
(*106*)	1978	Artificial contamination	Finger-tip broth culture	30	Plain soap < 4% CHG < P-I < 70% EA = alc. CHG
(*144*)	1978	Artificial contamination	Finger-tip broth culture	30	Plain soap < 4% CHG < 70% EA
(*107*)	1979	Existing hand flora	Hand-rub broth culture	120	Plain soap < 0.5% aq. CHG < 70% EA < 4% CHG < alc.CHG
(*145*)	1980	Artificial contamination	Finger-tip broth culture	60–120	4% CHG < P-I < 60% IPA
(*53*)	1980	Artificial contamination	Finger-tip broth culture	15	Plain soap < 3% HCP < P-I < 4% CHG < 70% EA
(*108*)	1982	Artificial contamination	Glove juice test	15	P-I < alc. CHG
(*109*)	1983	Artificial contamination	Finger-tip broth culture	120	0.3–2% triclosan = 60% IPA = alc. CHG < alc. triclosan
(*146*)	1984	Artificial contamination	Finger-tip agar culture	60	Phenolic < 4% CHG < P-I < EA < IPA < n-P
(*147*)	1985	Existing hand flora	Finger-tip agar culture	60	Plain soap < 70% EA < 95% EA
(*110*)	1986	Artificial contamination	Finger-tip broth culture	60	Phenolic = P-I < alc. CHG < n-P
(*93*)	1986	Existing hand flora	Sterile-broth bag technique	15	Plain soap < IPA < 4% CHG = IPA-E = alc. CHG
(*61*)	1988	Artificial contamination	Finger-tip broth culture	30	Plain soap < triclosan < P-I < IPA < alc. CHG < n-P
(*25*)	1991	Patient contact	Glove-juice test	15	Plain soap < IPA-E
(*148*)	1991	Existing hand flora	Agar-plate/image analysis	30	Plain soap < 1% triclosan < P-I < 4% CHG < IPA
(*111*)	1992	Artificial contamination	Finger-tip agar culture	60	Plain soap < IPA < EA < alc. CHG
(*149*)	1992	Artificial contamination	Finger-tip broth culture	60	Plain soap < 60% n-P
(*112*)	1994	Existing hand flora	Agar-plate/image analysis	30	Plain soap < alc. CHG
(*150*)	1999	Existing hand flora	Agar-plate culture	N.S.	Plain soap < commercial alcohol mixture
(*151*)	1999	Artificial contamination	Glove-juice test	20	Plain soap < 0.6% PCMX < 65% EA
(*152*)	1999	Artificial contamination	Finger-tip broth culture	30	4% CHG < plain soap < P-I < 70% EA

Note: Existing hand flora = without artificially contaminatiing hands with bacteria, alc. CHG = alcoholic chlorhexidine gluconate, aq. CHG = aqueous chlorhexidine gluconate, 4% CHG = chlorhexidine gluconate detergent, EA = ethanol, HCP = hexachlorophene soap/detergent, IPA = isopropanol, IPA-E = isopropanol + emollients, n-P = n-propanol, PCMX = chloroxylenol detergent, P-I = povidone-iodine detergent, and N.S. = not stated.

TABLE 4. Studies comparing the relative efficacy of plain soap or antimicrobial soap versus alcohol-containing products in reducing counts of bacteria recovered from hands immediately after use of products for pre-operative cleansing of hands

Ref. no.	Year	Assay method	Relative efficacy
(*143*)	1965	Finger-tip agar culture	HCP < 50% EA foam + QAC
(*157*)	1969	Finger-tip agar culture	HCP < P-I < 50% EA foam + QAC
(*101*)	1973	Finger-tip agar culture	HCP soap < EA foam + 0.23% HCP
(*135*)	1974	Broth culture	Plain soap < 0.5% CHG < 4% CHG < alc. CHG
(*119*)	1975	Hand-broth test	Plain soap < 0.5% CHG < 4% CHG < alc. CHG
(*118*)	1976	Glove-juice test	0.5% CHG < 4% CHG < alc. CHG
(*114*)	1977	Glove-juice test	P-I < CHG < alc. CHG
(*117*)	1978	Finger-tip agar culture	P-I = 46% EA + 0.23% HCP
(*113*)	1979	Broth culture of hands	Plain soap < P-I < alc. CHG < alc. P-I
(*116*)	1979	Glove-juice test	70% IPA = alc. CHG
(*147*)	1985	Finger-tip agar culture	Plain soap < 70% - 90% EA
(*115*)	1990	Glove-juice test, modified	Plain soap < triclosan < CHG < P-I < alc. CHG
(*104*)	1991	Glove-juice test	Plain soap < 2% triclosan < P-I < 70% IPA
(*158*)	1998	Finger-tip broth culture	70% IPA < 90% IPA = 60% n-P
(*159*)	1998	Glove-juice test	P-I < CHG < 70% EA

Note: QAC = quaternary ammonium compound, alc. CHG = alcoholic chlorhexidine gluconate, CHG = chlorhexidine gluconate detergent, EA = ethanol, HCP = hexachlorophene detergent, IPA = isopropanol, and P-I = povidone-iodine detergent.

TABLE 5. Efficacy of surgical hand-rub solutions in reducing the release of resident skin flora from clean hands

Study	Rub	Concentration* (%)	Time (min)	Mean log reduction	
				Immediate	Sustained (3 hr)
1	n-Propanol	60	5	2.9†	1.6†
2			5	2.7†	NA
3			5	2.5†	1.8†
4			5	2.3†	1.6†
5			3	2.9§	NA
4			3	2.0†	1.0†
4			1	1.1†	0.5†
6	Isopropanol	90	3	2.4§	1.4§
6		80	3	2.3§	1.2§
7		70	5	2.4†	2.1†
4			5	2.1†	1.0†
6			3	2.0§	0.7§
5			3	1.7¶	NA
4			3	1.5†	0.8†
8			2	1.2	0.8
4			1	0.7†	0.2
9			1	0.8	NA
10		60	5	1.7	1.0
7	Isopropanol + chlorhexidine gluc. (w/v)	70 + 0.5	5	2.5†	2.7†
8			2	1.0	1.5
11	Ethanol	95	2	2.1	NA
5		85	3	2.4§	NA
12		80	2	1.5	NA
8		70	2	1.0	0.6
13	Ethanol + chlorhexidine gluc. (w/v)	95 + 0.5	2	1.7	NA
14		77 + 0.5	5	2.0	1.5¶
8		70 + 0.5	2	0.7	1.4
8	Chlorhexidine gluc. (aq. Sol., w/v)	0.5	2	0.4	1.2
15	Povidone-iodine (aq. Sol., w/v)	1.0	5	1.9†	0.8†
16	Peracetic acid (w/v)	0.5	5	1.9	NA

Note: NA = not available.

Source: Rotter M. Hand washing and hand disinfection [Chapter 87]. In: Mayhall CG, ed. Hospital epidemiology and infection control. 2nd ed. Philadelphia, PA: Lippincott Williams & Wilkins, 1999. Table 5 is copyrighted by Lippincott Williams & Wilkins; it is reprinted here with their permission and permission from Manfred Rotler, M.D., Professor of Hygiene and Microbiology, Klinisches Institute für Hygiene der Universitat Wien, Germany.

* Volume/volume unless otherwise stated.

† Tested according to Deutsche Gesellschaft fur Hygiene, and Mikrobiologic (DGHM)-German Society of Hygiene and Microbiology method.

§ Tested according to European Standard prEN.

¶ After 4 hours.

and may vary for different formulations. However, if hands feel dry after rubbing hands together for 10–15 seconds, an insufficient volume of product likely was applied. Because alcohol-impregnated towelettes contain a limited amount of alcohol, their effectiveness is comparable to that of soap and water (*63,160,161*).

Alcohol-based hand rubs intended for use in hospitals are available as low viscosity rinses, gels, and foams. Limited data are available regarding the relative efficacy of various formulations. One field trial demonstrated that an ethanol gel was slightly more effective than a comparable ethanol solution at reducing bacterial counts on the hands of HCWs (*162*). However, a more recent study indicated that rinses reduced bacterial counts on the hands more than the gels tested (*80*). Further studies are warranted to determine the relative efficacy of alcohol-based rinses and gels in reducing transmission of health-care–associated pathogens.

Frequent use of alcohol-based formulations for hand antisepsis can cause drying of the skin unless emollients, humectants, or other skin-conditioning agents are added to the formulations. The drying effect of alcohol can be reduced or eliminated by adding 1%–3% glycerol or other skin-conditioning agents (*90,93,100,101,106,135,143,163,164*). Moreover, in several recent prospective trials, alcohol-based rinses or gels containing emollients caused substantially less skin irritation and dryness than the soaps or antimicrobial detergents tested (*96,98,165,166*). These studies, which were conducted in clinical settings, used various subjective and objective methods for assessing skin irritation and dryness. Further studies are warranted to establish whether products with different formulations yield similar results.

Even well-tolerated alcohol hand rubs containing emollients may cause a transient stinging sensation at the site of any broken skin (e.g., cuts and abrasions). Alcohol-based hand-rub preparations with strong fragrances may be poorly tolerated by HCWs with respiratory allergies. Allergic contact dermatitis or contact urticaria syndrome caused by hypersensitivity to alcohol or to various additives present in certain alcohol hand rubs occurs only rarely (*167,168*).

Alcohols are flammable. Flash points of alcohol-based hand rubs range from 21°C to 24°C, depending on the type and concentration of alcohol present (*169*). As a result, alcohol-based hand rubs should be stored away from high temperatures or flames in accordance with National Fire Protection Agency recommendations. In Europe, where alcohol-based hand rubs have been used extensively for years, the incidence of fires associated with such products has been low (*169*). One recent U.S. report described a flash fire that occurred as a result of an unusual series of events, which included an HCW applying an alcohol gel to her hands, immediately removing a polyester isolation gown, and then touching a metal door before the alcohol had evaporated (*170*). Removing the polyester gown created a substantial amount of static electricity that generated an audible static spark when the HCW touched the metal door, igniting the unevaporated alcohol on her hands (*170*). This incident emphasizes the need to rub hands together after application of alcohol-based products until all the alcohol has evaporated.

Because alcohols are volatile, containers should be designed to minimize evaporation. Contamination of alcohol-based solutions has seldom been reported. One report documented a cluster of pseudoinfections caused by contamination of ethyl alcohol by *Bacillus cereus* spores (*171*).

Chlorhexidine

Chlorhexidine gluconate, a cationic bisbiguanide, was developed in England in the early 1950s and was introduced into the United States in the 1970s (*8,172*). Chlorhexidine base is only minimally soluble in water, but the digluconate form is water-soluble. The antimicrobial activity of chlorhexidine is likely attributable to attachment to, and subsequent disruption of, cytoplasmic membranes, resulting in precipitation of cellular contents (*1,8*). Chlorhexidine's immediate antimicrobial activity occurs more slowly than that of alcohols. Chlorhexidine has good activity against gram-positive bacteria, somewhat less activity against gram-negative bacteria and fungi, and only minimal activity against tubercle bacilli (*1,8,172*). Chlorhexidine is not sporicidal (*1,172*). It has in vitro activity against enveloped viruses (e.g., herpes simplex virus, HIV, cytomegalovirus, influenza, and RSV) but substantially less activity against nonenveloped viruses (e.g., rotavirus, adenovirus, and enteroviruses) (*130,131,173*). The antimicrobial activity of chlorhexidine is only minimally affected by the presence of organic material, including blood. Because chlorhexidine is a cationic molecule, its activity can be reduced by natural soaps, various inorganic anions, nonionic surfactants, and hand creams containing anionic emulsifying agents (*8,172,174*). Chlorhexidine gluconate has been incorporated into a number of hand-hygiene preparations. Aqueous or detergent formulations containing 0.5% or 0.75% chlorhexidine are more effective than plain soap, but they are less effective than antiseptic detergent preparations containing 4% chlorhexidine gluconate (*135,175*). Preparations with 2% chlorhexidine gluconate are slightly less effective than those containing 4% chlorhexidine (*176*).

Chlorhexidine has substantial residual activity (*106,114–116,118,135,146,175*). Addition of low concentrations (0.5%–1.0%) of chlorhexidine to alcohol-based preparations results in greater residual activity than alcohol alone (*116,135*). When used as recommended, chlorhexidine has a good safety

record (*172*). Minimal, if any, absorption of the compound occurs through the skin. Care must be taken to avoid contact with the eyes when using preparations with ≥1% chlorhexidine, because the agent can cause conjunctivitis and severe corneal damage. Ototoxicity precludes its use in surgery involving the inner or middle ear. Direct contact with brain tissue and the meninges should be avoided. The frequency of skin irritation is concentration-dependent, with products containing 4% most likely to cause dermatitis when used frequently for antiseptic handwashing (*177*); allergic reactions to chlorhexidine gluconate are uncommon (*118,172*). Occasional outbreaks of nosocomial infections have been traced to contaminated solutions of chlorhexidine (*178–181*).

Chloroxylenol

Chloroxylenol, also known as parachlorometaxylenol (PCMX), is a halogen-substituted phenolic compound that has been used as a preservative in cosmetics and other products and as an active agent in antimicrobial soaps. It was developed in Europe in the late 1920s and has been used in the United States since the 1950s (*182*).

The antimicrobial activity of PCMX likely is attributable to inactivation of bacterial enzymes and alteration of cell walls (*1*). It has good in vitro activity against gram-positive organisms and fair activity against gram-negative bacteria, mycobacteria, and certain viruses (*1,7,182*). PCMX is less active against *P. aeruginosa*, but addition of ethylenediaminetetraacetic acid (EDTA) increases its activity against *Pseudomonas* spp. and other pathogens.

A limited number of articles focusing on the efficacy of PCMX-containing preparations intended for use by HCWs have been published in the last 25 years, and the results of studies have sometimes been contradictory. For example, in studies in which antiseptics were applied to abdominal skin, PCMX had the weakest immediate and residual activity of any of the agents studied (*183*). However, when 30-second handwashes were performed using 0.6% PCMX, 2% chlorhexidine gluconate, or 0.3% triclosan, the immediate effect of PCMX was similar to that of the other agents. When used 18 times per day for 5 consecutive days, PCMX had less cumulative activity than did chlorhexidine gluconate (*184*). When PCMX was used as a surgical scrub, one report indicated that 3% PCMX had immediate and residual activity comparable to 4% chlorhexidine gluconate (*185*), whereas two other studies demonstrated that the immediate and residual activity of PCMX was inferior to both chlorhexidine gluconate and povidone-iodine (*176,186*). The disparity between published studies may be associated with the various concentrations of PCMX included in the preparations evaluated and with other aspects of the formulations tested, including the presence or absence of EDTA (*7,182*). PCMX is not as rapidly active as chlorhexidine gluconate or iodophors, and its residual activity is less pronounced than that observed with chlorhexidine gluconate (*7,182*). In 1994, FDA TFM tentatively classified PCMX as a Category IIISE active agent (i.e., insufficient data are available to classify this agent as safe and effective) (*19*). Further evaluation of this agent by the FDA is ongoing.

The antimicrobial activity of PCMX is minimally affected by the presence of organic matter, but it is neutralized by nonionic surfactants. PCMX, which is absorbed through the skin (*7,182*), is usually well-tolerated, and allergic reactions associated with its use are uncommon. PCMX is available in concentrations of 0.3%–3.75%. In-use contamination of a PCMX-containing preparation has been reported (*187*).

Hexachlorophene

Hexachlorophene is a bisphenol composed of two phenolic groups and three chlorine moieties. In the 1950s and early 1960s, emulsions containing 3% hexachlorophene were widely used for hygienic handwashing, as surgical scrubs, and for routine bathing of infants in hospital nurseries. The antimicrobial activity of hexachlorophene results from its ability to inactivate essential enzyme systems in microorganisms. Hexachlorophene is bacteriostatic, with good activity against *S. aureus* and relatively weak activity against gram-negative bacteria, fungi, and mycobacteria (*7*).

Studies of hexachlorophene as a hygienic handwash and surgical scrub demonstrated only modest efficacy after a single handwash (*53,143,188*). Hexachlorophene has residual activity for several hours after use and gradually reduces bacterial counts on hands after multiple uses (i.e., it has a cumulative effect) (*1,101,188,189*). With repeated use of 3% hexachlorophene preparations, the drug is absorbed through the skin. Infants bathed with hexachlorophene and personnel regularly using a 3% hexachlorophene preparation for handwashing have blood levels of 0.1–0.6 ppm hexachlorophene (*190*). In the early 1970s, certain infants bathed with hexachlorophene developed neurotoxicity (vacuolar degeneration) (*191*). As a result, in 1972, the FDA warned that hexachlorophene should no longer be used routinely for bathing infants. However, after routine use of hexachlorophene for bathing infants in nurseries was discontinued, investigators noted that the incidence of health-care–associated *S. aureus* infections in hospital nurseries increased substantially (*192,193*). In several instances, the frequency of infections decreased when hexachlorophene bathing of infants was reinstituted. However, current guidelines still recommend against the routine bathing of neonates with hexachlorophene because of its potential neurotoxic effects (*194*). The agent is classified by FDA TFM as not

generally recognized as safe and effective for use as an antiseptic handwash (*19*). Hexachlorophene should not be used to bathe patients with burns or extensive areas of susceptible, sensitive skin. Soaps containing 3% hexachlorophene are available by prescription only (*7*).

Iodine and Iodophors

Iodine has been recognized as an effective antiseptic since the 1800s. However, because iodine often causes irritation and discoloring of skin, iodophors have largely replaced iodine as the active ingredient in antiseptics.

Iodine molecules rapidly penetrate the cell wall of microorganisms and inactivate cells by forming complexes with amino acids and unsaturated fatty acids, resulting in impaired protein synthesis and alteration of cell membranes (*195*). Iodophors are composed of elemental iodine, iodide or triiodide, and a polymer carrier (i.e., the complexing agent) of high molecular weight. The amount of molecular iodine present (so-called "free" iodine) determines the level of antimicrobial activity of iodophors. "Available" iodine refers to the total amount of iodine that can be titrated with sodium thiosulfate (*196*). Typical 10% povidone-iodine formulations contain 1% available iodine and yield free iodine concentrations of 1 ppm (*196*). Combining iodine with various polymers increases the solubility of iodine, promotes sustained release of iodine, and reduces skin irritation. The most common polymers incorporated into iodophors are polyvinyl pyrrolidone (i.e., povidone) and ethoxylated nonionic detergents (i.e., poloxamers) (*195,196*). The antimicrobial activity of iodophors also can be affected by pH, temperature, exposure time, concentration of total available iodine, and the amount and type of organic and inorganic compounds present (e.g., alcohols and detergents).

Iodine and iodophors have bactericidal activity against gram-positive, gram-negative, and certain spore-forming bacteria (e.g., clostridia and *Bacillus* spp.) and are active against mycobacteria, viruses, and fungi (*8,195,197–200*). However, in concentrations used in antiseptics, iodophors are not usually sporicidal (*201*). In vivo studies have demonstrated that iodophors reduce the number of viable organisms that are recovered from the hands of personnel (*113,145,148,152,155*). Povidone-iodine 5%–10% has been tentatively classified by FDA TFM as a Category I agent (i.e., a safe and effective agent for use as an antiseptic handwash and an HCW handwash) (*19*). The extent to which iodophors exhibit persistent antimicrobial activity after they have been washed off the skin is unclear. In one study, persistent activity was noted for 6 hours (*176*); however, several other studies demonstrated persistent activity for only 30–60 minutes after washing hands with an iodophor (*61,117,202*). In studies in which bacterial counts were obtained after gloves were worn for 1–4 hours after washing, iodophors have demonstrated poor persistent activity (*1,104,115,189,203–208*). The in vivo antimicrobial activity of iodophors is substantially reduced in the presence of organic substances (e.g., blood or sputum) (*8*).

The majority of iodophor preparations used for hand hygiene contain 7.5%–10% povidone-iodine. Formulations with lower concentrations also have good antimicrobial activity because dilution can increase free iodine concentrations (*209*). However, as the amount of free iodine increases, the degree of skin irritation also may increase (*209*). Iodophors cause less skin irritation and fewer allergic reactions than iodine, but more irritant contact dermatitis than other antiseptics commonly used for hand hygiene (*92*). Occasionally, iodophor antiseptics have become contaminated with gram-negative bacilli as a result of poor manufacturing processes and have caused outbreaks or pseudo-outbreaks of infection (*196*).

Quaternary Ammonium Compounds

Quaternary ammonium compounds are composed of a nitrogen atom linked directly to four alkyl groups, which may vary in their structure and complexity (*210*). Of this large group of compounds, alkyl benzalkonium chlorides are the most widely used as antiseptics. Other compounds that have been used as antiseptics include benzethonium chloride, cetrimide, and cetylpyridium chloride (*1*). The antimicrobial activity of these compounds was first studied in the early 1900s, and a quaternary ammonium compound for preoperative cleaning of surgeons' hands was used as early as 1935 (*210*). The antimicrobial activity of this group of compounds likely is attributable to adsorption to the cytoplasmic membrane, with subsequent leakage of low molecular weight cytoplasmic constituents (*210*).

Quaternary ammonium compounds are primarily bacteriostatic and fungistatic, although they are microbicidal against certain organisms at high concentrations (*1*); they are more active against gram-positive bacteria than against gram-negative bacilli. Quaternary ammonium compounds have relatively weak activity against mycobacteria and fungi and have greater activity against lipophilic viruses. Their antimicrobial activity is adversely affected by the presence of organic material, and they are not compatible with anionic detergents (*1,210*). In 1994, FDA TFM tentatively classified benzalkonium chloride and benzethonium chloride as Category IIISE active agents (i.e., insufficient data exists to classify them as safe and effective for use as an antiseptic handwash) (*19*). Further evaluation of these agents by FDA is in progress.

Quaternary ammonium compounds are usually well tolerated. However, because of weak activity against

gram-negative bacteria, benzalkonium chloride is prone to contamination by these organisms. Several outbreaks of infection or pseudoinfection have been traced to quaternary ammonium compounds contaminated with gram-negative bacilli (*211–213*). For this reason, in the United States, these compounds have been seldom used for hand antisepsis during the last 15–20 years. However, newer handwashing products containing benzalkonium chloride or benzethonium chloride have recently been introduced for use by HCWs. A recent study of surgical intensive-care unit personnel found that cleaning hands with antimicrobial wipes containing a quaternary ammonium compound was about as effective as using plain soap and water for handwashing; both were less effective than decontaminating hands with an alcohol-based hand rub (*214*). One laboratory-based study reported that an alcohol-free hand-rub product containing a quaternary ammonium compound was efficacious in reducing microbial counts on the hands of volunteers (*215*). Further studies of such products are needed to determine if newer formulations are effective in health-care settings.

Triclosan

Triclosan (chemical name: 2,4,4' –trichloro-2'-hydroxydiphenyl ether) is a nonionic, colorless substance that was developed in the 1960s. It has been incorporated into soaps for use by HCWs and the public and into other consumer products. Concentrations of 0.2%–2% have antimicrobial activity. Triclosan enters bacterial cells and affects the cytoplasmic membrane and synthesis of RNA, fatty acids, and proteins (*216*). Recent studies indicate this agent's antibacterial activity is attributable to binding to the active site of enoyl-acyl carrier protein reductase (*217,218*).

Triclosan has a broad range of antimicrobial activity, but it is often bacteriostatic (*1*). Minimum inhibitory concentrations (MICs) range from 0.1 to 10 ug/mL, whereas minimum bactericidal concentrations are 25–500 ug/mL. Triclosan's activity against gram-positive organisms (including MRSA) is greater than against gram-negative bacilli, particularly *P. aeruginosa* (*1,216*). The agent possesses reasonable activity against mycobacterial and *Candida* spp., but it has limited activity against filamentous fungi. Triclosan (0.1%) reduces bacterial counts on hands by 2.8 $\log_{10}$ after a 1-minute hygienic handwash (*1*). In several studies, log reductions have been lower after triclosan is used than when chlorhexidine, iodophors, or alcohol-based products are applied (*1,61,149,184,219*). In 1994, FDA TFM tentatively classified triclosan ≤1.0% as a Category IIISE active agent (i.e., insufficient data exist to classify this agent as safe and effective for use as an antiseptic handwash) (*19*). Further evaluation of this agent by the FDA is underway. Like chlorhexidine, triclosan has persistent activity on the skin. Its activity in hand-care products is affected by pH, the presence of surfactants, emollients, or humectants and by the ionic nature of the particular formulation (*1,216*). Triclosan's activity is not substantially affected by organic matter, but it can be inhibited by sequestration of the agent in micelle structures formed by surfactants present in certain formulations. The majority of formulations containing <2% triclosan are well-tolerated and seldom cause allergic reactions. Certain reports indicate that providing hospital personnel with a triclosan-containing preparation for hand antisepsis has led to decreased MRSA infections (*72,73*). Triclosan's lack of potent activity against gram-negative bacilli has resulted in occasional reports of contamination (*220*).

Other Agents

Approximately 150 years after puerperal-fever–related maternal mortality rates were demonstrated by Semmelweis to be reduced by use of a hypochlorite hand rinse, the efficacy of rubbing hands for 30 seconds with an aqueous hypochlorite solution was studied once again (*221*). The solution was demonstrated to be no more effective than distilled water. The regimen used by Semmelweis, which called for rubbing hands with a 4% [w/w] hypochlorite solution until the hands were slippery (approximately 5 minutes), has been revisited by other researchers (*222*). This more current study indicated that the regimen was 30 times more effective than a 1-minute rub using 60% isopropanol. However, because hypochlorite solutions are often irritating to the skin when used repeatedly and have a strong odor, they are seldom used for hand hygiene.

Certain other agents are being evaluated by FDA for use in health-care-related antiseptics (*19*). However, the efficacy of these agents has not been evaluated adequately for use in handwashing preparations intended for use by HCWs. Further evaluation of these agents is warranted. Products that use different concentrations of traditional antiseptics (e.g., low concentrations of iodophor) or contain novel compounds with antiseptic properties are likely to be introduced for use by HCWs. For example, preliminary studies have demonstrated that adding silver-containing polymers to an ethanol carrier (i.e., Surfacine®) results in a preparation that has persistent antimicrobial activity on animal and human skin (*223*). New compounds with good in vitro activity must be tested in vivo to determine their abilities to reduce transient and resident skin flora on the hands of HCWs.

Activity of Antiseptic Agents Against Spore-Forming Bacteria

The widespread prevalence of health-care–associated diarrhea caused by *Clostridium difficile* and the recent occurrence

in the United States of human *Bacillus anthracis* infections associated with contaminated items sent through the postal system has raised concern regarding the activity of antiseptic agents against spore-forming bacteria. None of the agents (including alcohols, chlorhexidine, hexachlorophene, iodophors, PCMX, and triclosan) used in antiseptic handwash or antiseptic hand-rub preparations are reliably sporicidal against *Clostridium* spp. or *Bacillus* spp. (*120,172,224,225*). Washing hands with non-antimicrobial or antimicrobial soap and water may help to physically remove spores from the surface of contaminated hands. HCWs should be encouraged to wear gloves when caring for patients with *C. difficile*-associated diarrhea (*226*). After gloves are removed, hands should be washed with a non-antimicrobial or an antimicrobial soap and water or disinfected with an alcohol-based hand rub. During outbreaks of *C. difficile*-related infections, washing hands with a non-antimicrobial or antimicrobial soap and water after removing gloves is prudent. HCWs with suspected or documented exposure to *B. anthracis*-contaminated items also should be encouraged to wash their hands with a non-antimicrobial or antimicrobial soap and water.

Reduced Susceptibility of Bacteria to Antiseptics

Reduced susceptibility of bacteria to antiseptic agents can either be an intrinsic characteristic of a species or can be an acquired trait (*227*). Several reports have described strains of bacteria that appear to have acquired reduced susceptibility (when defined by MICs established in vitro) to certain antiseptics (e.g., chlorhexidine, quaternary ammonium compounds, and triclosan) (*227–230*). However, because the antiseptic concentrations that are actually used by HCWs are often substantially higher than the MICs of strains with reduced antiseptic susceptibility, the clinical relevance of the in vitro findings is questionable. For example, certain strains of MRSA have chlorhexidine and quaternary ammonium compound MICs that are several-fold higher than methicillin-susceptible strains, and certain strains of *S. aureus* have elevated MICs to triclosan (*227,228*). However, such strains were readily inhibited by the concentrations of these antiseptics that are actually used by practicing HCWs (*227,228*). The description of a triclosan-resistant bacterial enzyme has raised the question of whether resistance to this agent may develop more readily than to other antiseptic agents (*218*). In addition, exposing *Pseudomonas* strains containing the MexAB-OprM efflux system to triclosan may select for mutants that are resistant to multiple antibiotics, including fluoroquinolones (*230*). Further studies are needed to determine whether reduced susceptibility to antiseptic agents is of epidemiologic significance and whether resistance to antiseptics has any influence on the prevalence of antibiotic-resistant strains (*227*).

Surgical Hand Antisepsis

Since the late 1800s, when Lister promoted the application of carbolic acid to the hands of surgeons before procedures, preoperative cleansing of hands and forearms with an antiseptic agent has been an accepted practice (*231*). Although no randomized, controlled trials have been conducted to indicate that surgical-site infection rates are substantially lower when preoperative scrubbing is performed with an antiseptic agent rather than a non-antimicrobial soap, certain other factors provide a strong rationale for this practice. Bacteria on the hands of surgeons can cause wound infections if introduced into the operative field during surgery (*232*); rapid multiplication of bacteria occurs under surgical gloves if hands are washed with a non-antimicrobial soap. However, bacterial growth is slowed after preoperative scrubbing with an antiseptic agent (*14,233*). Reducing resident skin flora on the hands of the surgical team for the duration of a procedure reduces the risk of bacteria being released into the surgical field if gloves become punctured or torn during surgery (*1,156,169*). Finally, at least one outbreak of surgical-site infections occurred when surgeons who normally used an antiseptic surgical scrub preparation began using a non-antimicrobial product (*234*).

Antiseptic preparations intended for use as surgical hand scrubs are evaluated for their ability to reduce the number of bacteria released from hands at different times, including 1) immediately after scrubbing, 2) after wearing surgical gloves for 6 hours (i.e., persistent activity), and 3) after multiple applications over 5 days (i.e., cumulative activity). Immediate and persistent activity are considered the most important in determining the efficacy of the product. U.S. guidelines recommend that agents used for surgical hand scrubs should substantially reduce microorganisms on intact skin, contain a nonirritating antimicrobial preparation, have broad-spectrum activity, and be fast-acting and persistent (*19,235*).

Studies have demonstrated that formulations containing 60%–95% alcohol alone or 50%–95% when combined with limited amounts of a quaternary ammonium compound, hexachlorophene, or chlorhexidine gluconate, lower bacterial counts on the skin immediately postscrub more effectively than do other agents (Table 4). The next most active agents (in order of decreasing activity) are chlorhexidine gluconate, iodophors, triclosan, and plain soap (*104,119,186,188, 203,204,206,208,236*). Because studies of PCMX as a surgical scrub have yielded contradictory results, further studies are needed to establish how the efficacy of this compound compares with the other agents (*176,185,186*).

Although alcohols are not considered to have persistent antimicrobial activity, bacteria appear to reproduce slowly on the hands after a surgical scrub with alcohol, and bacterial counts on hands after wearing gloves for 1–3 hours seldom exceed baseline (i.e., prescrub) values (*1*). However, a recent study demonstrated that a formulation containing 61% ethanol alone did not achieve adequate persistent activity at 6 hours postscrub (*237*). Alcohol-based preparations containing 0.5% or 1% chlorhexidine gluconate have persistent activity that, in certain studies, has equaled or exceeded that of chlorhexidine gluconate-containing detergents (*1,118,135,237*).*

Persistent antimicrobial activity of detergent-based surgical scrub formulations is greatest for those containing 2% or 4% chlorhexidine gluconate, followed by hexachlorophene, triclosan, and iodophors (*1,102,113–115,159,189,203, 204,206–208,236*). Because hexachlorophene is absorbed into the blood after repeated use, it is seldom used as a surgical scrub.

Surgical staff have been traditionally required to scrub their hands for 10 minutes preoperatively, which frequently leads to skin damage. Several studies have demonstrated that scrubbing for 5 minutes reduces bacterial counts as effectively as a 10-minute scrub (*117,238,239*). In other studies, scrubbing for 2 or 3 minutes reduced bacterial counts to acceptable levels (*156,205,207,240,241*).

Studies have indicated that a two-stage surgical scrub using an antiseptic detergent, followed by application of an alcohol-containing preparation, is effective. For example, an initial 1- or 2-minute scrub with 4% chlorhexidine gluconate or povidone-iodine followed by application of an alcohol-based product has been as effective as a 5-minute scrub with an antiseptic detergent (*114,242*).

Surgical hand-antisepsis protocols have required personnel to scrub with a brush. But this practice can damage the skin of personnel and result in increased shedding of bacteria from the hands (*95,243*). Scrubbing with a disposable sponge or combination sponge-brush has reduced bacterial counts on the hands as effectively as scrubbing with a brush (*244–246*). However, several studies indicate that neither a brush nor a sponge is necessary to reduce bacterial counts on the hands of surgical personnel to acceptable levels, especially when alcohol-based products are used (*102,117,159,165,233,237, 247,248*). Several of these studies performed cultures immediately or at 45–60 minutes postscrub (*102,117, 233,247,248*), whereas in other studies, cultures were obtained 3 and 6 hours postscrub (*159,237*). For example, a recent laboratory-based study using volunteers demonstrated that brushless application of a preparation containing 1% chlorhexidine gluconate plus 61% ethanol yielded lower bacterial counts on the hands of participants than using a sponge/brush to apply a 4% chlorhexidine-containing detergent preparation (*237*).

Relative Efficacy of Plain Soap, Antiseptic Soap/Detergent, and Alcohols

Comparing studies related to the in vivo efficacy of plain soap, antimicrobial soaps, and alcohol-based hand rubs is problematic, because certain studies express efficacy as the percentage reduction in bacterial counts achieved, whereas others give $\log_{10}$ reductions in counts achieved. However, summarizing the relative efficacy of agents tested in each study can provide an overview of the in vivo activity of various formulations intended for handwashing, hygienic handwash, antiseptic hand rub, or surgical hand antisepsis (Tables 2–4).

Irritant Contact Dermatitis Resulting from Hand-Hygiene Measures

Frequency and Pathophysiology of Irritant Contact Dermatitis

In certain surveys, approximately 25% of nurses report symptoms or signs of dermatitis involving their hands, and as many as 85% give a history of having skin problems (*249*). Frequent and repeated use of hand-hygiene products, particularly soaps and other detergents, is a primary cause of chronic irritant contact dermatitis among HCWs (*250*). The potential of detergents to cause skin irritation can vary considerably and can be ameliorated by the addition of emollients and humectants. Irritation associated with antimicrobial soaps may be caused by the antimicrobial agent or by other ingredients of the formulation. Affected persons often complain of a feeling of dryness or burning; skin that feels "rough;" and erythema, scaling, or fissures. Detergents damage the skin by causing denaturation of stratum corneum proteins, changes in intercellular lipids (either depletion or reorganization of lipid moieties), decreased corneocyte cohesion, and decreased stratum corneum water-binding capacity (*250,251*). Damage

* In a recent randomized clinical trial, surgical site infection rates were monitored among patients who were operated on by surgical personnel who cleaned their hands preoperatively either by performing a traditional 5-minute surgical hand scrub using 4% povidone-iodine or 4% antisepsis antimicrobial soap, or by washing their hands for 1 minute with a non-antimicrobial soap followed by a 5-minute hand-rubbing technique using an alcohol-based hand rinse containing 0.2% mecetronium etilsulfate. The incidence of surgical site infections was virtually identical in the two groups of patients. (Source: Parienti JJ, Thibon P, Heller R, et al. for Members of the Antisepsie Chirurgicale des Mains Study Group. Hand-rubbing with an aqueous alcoholic solution vs traditional surgical hand-scrubbing and 30-day surgical site infection rates: a randomized equivalence study. JAMA 2002;288:722–7).

to the skin also changes skin flora, resulting in more frequent colonization by staphylococci and gram-negative bacilli (*17,90*). Although alcohols are among the safest antiseptics available, they can cause dryness and irritation of the skin (*1,252*). Ethanol is usually less irritating than n-propanol or isopropanol (*252*).

Irritant contact dermatitis is more commonly reported with iodophors (*92*). Other antiseptic agents that can cause irritant contact dermatitis (in order of decreasing frequency) include chlorhexidine, PCMX, triclosan, and alcohol-based products. Skin that is damaged by repeated exposure to detergents may be more susceptible to irritation by alcohol-based preparations (*253*). The irritancy potential of commercially prepared hand-hygiene products, which is often determined by measuring transepidermal water loss, may be available from the manufacturer. Other factors that can contribute to dermatitis associated with frequent handwashing include using hot water for handwashing, low relative humidity (most common in winter months), failure to use supplementary hand lotion or cream, and the quality of paper towels (*254,255*). Shear forces associated with wearing or removing gloves and allergy to latex proteins may also contribute to dermatitis of the hands of HCWs.

Allergic Contact Dermatitis Associated with Hand-Hygiene Products

Allergic reactions to products applied to the skin (i.e., contact allergies) may present as delayed type reactions (i.e., allergic contact dermatitis) or less commonly as immediate reactions (i.e., contact urticaria). The most common causes of contact allergies are fragrances and preservatives; emulsifiers are less common causes (*256–259*). Liquid soaps, hand lotions or creams, and "udder ointments" may contain ingredients that cause contact allergies among HCWs (*257,258*).

Allergic reactions to antiseptic agents, including quaternary ammonium compounds, iodine or iodophors, chlorhexidine, triclosan, PCMX, and alcohols have been reported (*118,167,172,256,260–265*). Allergic contact dermatitis associated with alcohol-based hand rubs is uncommon. Surveillance at a large hospital in Switzerland, where a commercial alcohol hand rub has been used for >10 years, failed to identify a single case of documented allergy to the product (*169*). In late 2001, a Freedom of Information Request for data in the FDA's Adverse Event Reporting System regarding adverse reactions to popular alcohol hand rubs in the United States yielded only one reported case of an erythematous rash reaction attributed to such a product (John M. Boyce, M.D., Hospital of St. Raphael, New Haven, Connecticut, personal communication, 2001). However, with increasing use of such products by HCWs, true allergic reactions to such products likely will be encountered.

Allergic reactions to alcohol-based products may represent true allergy to alcohol, allergy to an impurity or aldehyde metabolite, or allergy to another constituent of the product (*167*). Allergic contact dermatitis or immediate contact urticarial reactions may be caused by ethanol or isopropanol (*167*). Allergic reactions can be caused by compounds that may be present as inactive ingredients in alcohol-based hand rubs, including fragrances, benzyl alcohol, stearyl or isostearyl alcohol, phenoxyethanol, myristyl alcohol, propylene glycol, parabens, and benzalkonium chloride (*167,256,266–270*).

Proposed Methods for Reducing Adverse Effects of Agents

Potential strategies for minimizing hand-hygiene–related irritant contact dermatitis among HCWs include reducing the frequency of exposure to irritating agents (particularly anionic detergents), replacing products with high irritation potential with preparations that cause less damage to the skin, educating personnel regarding the risks of irritant contact dermatitis, and providing caregivers with moisturizing skin-care products or barrier creams (*96,98,251,271–273*). Reducing the frequency of exposure of HCWs to hand-hygiene products would prove difficult and is not desirable because of the low levels of adherence to hand-hygiene policies in the majority of institutions. Although hospitals have provided personnel with non-antimicrobial soaps in hopes of minimizing dermatitis, frequent use of such products may cause greater skin damage, dryness, and irritation than antiseptic preparations (*92,96,98*). One strategy for reducing the exposure of personnel to irritating soaps and detergents is to promote the use of alcohol-based hand rubs containing various emollients. Several recent prospective, randomized trials have demonstrated that alcohol-based hand rubs containing emollients were better tolerated by HCWs than washing hands with non-antimicrobial soaps or antimicrobial soaps (*96,98,166*). Routinely washing hands with soap and water immediately after using an alcohol hand rub may lead to dermatitis. Therefore, personnel should be reminded that it is neither necessary nor recommended to routinely wash hands after each application of an alcohol hand rub.

Hand lotions and creams often contain humectants and various fats and oils that can increase skin hydration and replace altered or depleted skin lipids that contribute to the barrier function of normal skin (*251,271*). Several controlled trials have demonstrated that regular use (e.g., twice a day) of such products can help prevent and treat irritant contact dermatitis caused by hand-hygiene products (*272,273*). In one study, frequent and scheduled use of an oil-containing lotion improved skin condition, and thus led to a 50% increase in

handwashing frequency among HCWs (273). Reports from these studies emphasize the need to educate personnel regarding the value of regular, frequent use of hand-care products.

Recently, barrier creams have been marketed for the prevention of hand-hygiene–related irritant contact dermatitis. Such products are absorbed to the superficial layers of the epidermis and are designed to form a protective layer that is not removed by standard handwashing. Two recent randomized, controlled trials that evaluated the skin condition of caregivers demonstrated that barrier creams did not yield better results than did the control lotion or vehicle used (*272,273*). As a result, whether barrier creams are effective in preventing irritant contact dermatitis among HCWs remains unknown.

In addition to evaluating the efficacy and acceptability of hand-care products, product-selection committees should inquire about the potential deleterious effects that oil-containing products may have on the integrity of rubber gloves and on the efficacy of antiseptic agents used in the facility (*8,236*).

Factors To Consider When Selecting Hand-Hygiene Products

When evaluating hand-hygiene products for potential use in health-care facilities, administrators or product-selection committees must consider factors that can affect the overall efficacy of such products, including the relative efficacy of antiseptic agents against various pathogens (Appendix) and acceptance of hand-hygiene products by personnel (*274,275*). Soap products that are not well-accepted by HCWs can be a deterrent to frequent handwashing (*276*). Characteristics of a product (either soap or alcohol-based hand rub) that can affect acceptance by personnel include its smell, consistency (i.e., "feel"), and color (*92,277,278*). For soaps, ease of lathering also may affect user preference.

Because HCWs may wash their hands from a limited number of times per shift to as many as 30 times per shift, the tendency of products to cause skin irritation and dryness is a substantial factor that influences acceptance, and ultimate usage (*61,98,274,275,277,279*). For example, concern regarding the drying effects of alcohol was a primary cause of poor acceptance of alcohol-based hand-hygiene products in hospitals in the United States (*5,143*). However, several studies have demonstrated that alcohol-based hand rubs containing emollients are acceptable to HCWs (*90,93,98,100,101,106, 143,163,164,166*). With alcohol-based products, the time required for drying may also affect user acceptance.

Studies indicate that the frequency of handwashing or antiseptic handwashing by personnel is affected by the accessibility of hand-hygiene facilities (*280–283*). In certain health-care facilities, only one sink is available in rooms housing several patients, or sinks are located far away from the door of the room, which may discourage handwashing by personnel leaving the room. In intensive-care units, access to sinks may be blocked by bedside equipment (e.g., ventilators or intravenous infusion pumps). In contrast to sinks used for handwashing or antiseptic handwash, dispensers for alcohol-based hand rubs do not require plumbing and can be made available adjacent to each patient's bed and at many other locations in patient-care areas. Pocket carriage of alcohol-based hand-rub solutions, combined with availability of bedside dispensers, has been associated with substantial improvement in adherence to hand-hygiene protocols (*74,284*). To avoid any confusion between soap and alcohol hand rubs, alcohol hand-rub dispensers should not be placed adjacent to sinks. HCWs should be informed that washing hands with soap and water after each use of an alcohol hand rub is not necessary and is not recommended, because it may lead to dermatitis. However, because personnel feel a "build-up" of emollients on their hands after repeated use of alcohol hand gels, washing hands with soap and water after 5–10 applications of a gel has been recommended by certain manufacturers.

Automated handwashing machines have not been demonstrated to improve the quality or frequency of handwashing (*88,285*). Although technologically advanced automated handwashing devices and monitoring systems have been developed recently, only a minimal number of studies have been published that demonstrate that use of such devices results in enduring improvements in hand-hygiene adherence among HCWs. Further evaluation of automated handwashing facilities and monitoring systems is warranted.

Dispenser systems provided by manufacturers or vendors also must be considered when evaluating hand-hygiene products. Dispensers may discourage use by HCWs when they 1) become blocked or partially blocked and do not deliver the product when accessed by personnel, and 2) do not deliver the product appropriately onto the hands. In one hospital where a viscous alcohol-based hand rinse was available, only 65% of functioning dispensers delivered product onto the caregivers' hands with one press of the dispenser lever, and 9% of dispensers were totally occluded (*286*). In addition, the volume delivered was often suboptimal, and the product was sometimes squirted onto the wall instead of the caregiver's hand.

Only limited information is available regarding the cost of hand-hygiene products used in health-care facilities (*165,287*). These costs were evaluated in patient-care areas at a 450-bed community teaching hospital (*287*); the hospital spent $22,000 ($0.72 per patient-day) on 2% chlorhexidine-containing preparations, plain soap, and an alcohol hand rinse. *(287)* When

hand-hygiene supplies for clinics and nonpatient care areas were included, the total annual budget for soaps and hand antiseptic agents was $30,000 (approximately $1 per patient-day). Annual hand-hygiene product budgets at other institutions vary considerably because of differences in usage patterns and varying product prices. One researcher (*287*) determined that if non-antimicrobial liquid soap were assigned an arbitrary relative cost of 1.0, the cost per liter would be 1.7 times as much for 2% chlorhexidine gluconate detergent, 1.6–2.0 times higher for alcohol-based hand-rub products, and 4.5 times higher for an alcohol-based foam product. A recent cost comparison of surgical scrubbing with an antimicrobial soap versus brushless scrubbing with an alcohol-based hand rub revealed that costs and time required for preoperative scrubbing were less with the alcohol-based product (*165*). In a trial conducted in two critical-care units, the cost of using an alcohol hand rub was half as much as using an antimicrobial soap for handwashing ($0.025 versus $0.05 per application, respectively) (*166*).

To put expenditures for hand-hygiene products into perspective, health-care facilities should consider comparing their budget for hand-hygiene products to estimated excess hospital costs resulting from health-care–associated infections. The excess hospital costs associated with only four or five health-care–associated infections of average severity may equal the entire annual budget for hand-hygiene products used in inpatient-care areas. Just one severe surgical site infection, lower respiratory tract infection, or bloodstream infection may cost the hospital more than the entire annual budget for antiseptic agents used for hand hygiene (*287*). Two studies provided certain quantitative estimates of the benefit of hand-hygiene–promotion programs (*72,74*). One study demonstrated a cost saving of approximately $17,000 resulting from reduced use of vancomycin after the observed decrease in MRSA incidence in a 7-month period (*72*). In another study that examined both direct costs associated with the hand-hygiene promotion program (increased use of hand-rub solution and poster production) and indirect costs associated with health-care–personnel time (*74*), costs of the program were an estimated $57,000 or less per year (an average of $1.42 per patient admitted). Supplementary costs associated with the increased use of alcohol-based hand-rub solution averaged $6.07 per 100 patient-days. Based on conservative estimates of $2,100 saved per infection averted and on the assumption that only 25% of the observed reduction in the infection rate was associated with improved hand-hygiene practice, the program was substantially cost-effective. Thus, hospital administrators must consider that by purchasing more effective or more acceptable hand-hygiene products to improve hand-hygiene practices, they will avoid the occurrence of nosocomial infections; preventing only a limited number of additional health-care–associated infections per year will lead to savings that will exceed any incremental costs of improved hand-hygiene products.

Hand-Hygiene Practices Among HCWs

In observational studies conducted in hospitals, HCWs washed their hands an average of five times per shift to as many as 30 times per shift (Table 6) (*17,61,90,98,274,288*); certain nurses washed their hands ≤100 times per shift (*90*). Hospitalwide surveillance of hand hygiene reveals that the average number of handwashing opportunities varies markedly between hospital wards. For example, nurses in pediatric wards had an average of eight opportunities for hand hygiene per hour of patient care compared with an average of 20 for nurses in intensive-care units (*11*). The duration of handwashing or hygienic handwash episodes by HCWs has averaged 6.6–24.0 seconds in observational studies (Table 7) (*17,52,59,84–87,89,249,279*). In addition to washing their

TABLE 6. Handwashing frequency among health-care workers

Ref. no.	Year	Avg. no./ time period	Range	Avg. no./hr
(*61*)	1988	5/8 hour	N.S.	
(*89*)	1984	5–10/shift	N.S.	
(*96*)	2000	10/shift	N.S.	
(*273*)	2000	12–18/day	2–60	
(*98*)	2000	13–15/8 hours	5–27	1.6–1.8/hr
(*90*)	1977	20–42/8 hours	10–100	
(*391*)	2000	21/12 hours	N.S.	
(*272*)	2000	22/day	0–70	
(*88*)	1991			1.7–2.1/hr
(*17*)	1998			2.1/hr
(*279*)	1978			3/hr
(*303*)	1994			3.3/hr

Note: N.S. = Not Stated.

TABLE 7. Average duration of handwashing by health-care workers

Ref. no.	Year	Mean/median time
(*392*)	1997	4.7–5.3 seconds
(*303*)	1994	6.6 seconds
(*52*)	1974	8–9.3 seconds
(*85*)	1984	8.6 seconds
(*86*)	1994	<9 seconds
(*87*)	1994	9.5 seconds
(*88*)	1991	<10 seconds
(*294*)	1990	10 seconds
(*89*)	1984	11.6 seconds
(*300*)	1992	12.5 seconds
(*59*)	1988	15.6–24.4 seconds
(*17*)	1998	20.6 seconds
(*279*)	1978	21 seconds
(*293*)	1989	24 seconds

hands for limited time periods, personnel often fail to cover all surfaces of their hands and fingers (*288*).

Adherence of HCWs to Recommended Hand-Hygiene Practices

Observational Studies of Hand-Hygiene Adherence. Adherence of HCWs to recommended hand-hygiene procedures has been poor, with mean baseline rates of 5%–81% (overall average: 40%) (Table 8) (*71,74,86,87,276,280,281,283,285,289–313*). The methods used for defining adherence (or nonadherence) and those used for conducting observations vary considerably among studies, and reports do not provide detailed information concerning the methods and criteria used. The majority of studies were conducted with hand-hygiene adherence as the major outcome measure, whereas a limited number measured adherence as part of a broader investigation. Several investigators reported improved adherence after implementing various interventions, but the majority of studies had short follow-up periods and did not confirm whether behavioral improvements were long-lasting. Other studies established that sustained improvements in handwashing behavior occurred during a long-term program to improve adherence to hand-hygiene policies (*74,75*).

TABLE 8. Hand-hygiene adherence by health-care workers (1981–2000)

Ref. no.	Year	Setting	Before/ after	Adherence baseline	Adherence after intervention	Invervention
(*280*)	1981	ICU	A	16%	30%	More convenient sink locations
(*289*)	1981	ICU	A	41%	—	
		ICU	A	28%	—	
(*290*)	1983	All wards	A	45%	—	
(*281*)	1986	SICU	A	51%	—	
		MICU	A	76%	—	
(*276*)	1986	ICU	A	63%	92%	Performance feedback
(*291*)	1987	PICU	A	31%	30%	Wearing overgown
(*292*)	1989	MICU	B/A	14%/28%*	73%/81%	Feedback, policy reviews, memo, and posters
		MICU	B/A	26%/23%	38%/60%	
(*293*)	1989	NICU	A/B	75%/50%	—	
(*294*)	1990	ICU	A	32%	45%	Alcohol rub introduced
(*295*)	1990	ICU	A	81%	92%	Inservices first, then group feedback
(*296*)	1990	ICU	B/A	22%	30%	
(*297*)	1991	SICU	A	51%	—	
(*298*)	1991	Pedi OPDs	B	49%	49%	Signs, feedback, and verbal reminders to physicians
(*299*)	1991	Nursery and NICU	B/A†	28%	63%	Feedback, dissemination of literature, and results of environmental cultures
(*300*)	1992	NICU/others	A	29%	—	
(*71*)	1992	ICU	N.S.	40%	—	
(*301*)	1993	ICUs	A	40%	—	
(*87*)	1994	Emergency Room	A	32%	—	
(*86*)	1994	All wards	A	32%	—	
(*285*)	1994	SICU	A	22%	38%	Automated handwashing machines available
(*302*)	1994	NICU	A	62%	60%	No gowning required
(*303*)	1994	ICU Wards	AA	30%29%	—	
(*304*)	1995	ICU Oncol Ward	A	56%	—	
(*305*)	1995	ICU	N.S.	5%	63%	Lectures, feedback, and demonstrations
(*306*)	1996	PICU	B/A	12%/11%	68%/65%	Overt observation, followed by feedback
(*307*)	1996	MICU	A	41%	58%	Routine wearing of gowns and gloves
(*308*)	1996	Emergency Dept	A	54%	64%	Signs/distributed review paper
(*309*)	1998	All wards	A	30%	—	
(*310*)	1998	Pediatric wards	B/A	52%/49%	74%/69%	Feedback, movies, posters, and brochures
(*311*)	1999	MICU	B/A	12%/55%	—	
(*74*)	2000	All wards	B/A	48%	67%	Posters, feedback, administrative support, and alcohol rub
(*312*)	2000	MICU	A	42%	61%	Alcohol hand rub made available
(*283*)	2000	MICU	B/A	10%/22%	23%/48%	Education, feedback, and alcohol gel made available
		CTICU	B/A	4%/13%	7%/14%	
(*313*)	2000	Medical wards	A	60%	52%	Education, reminders, and alcohol gel made available

Note: ICU = intensive care unit, SICU = surgical ICU, MICU = medical ICU, PICU = pediatric ICU, NICU = neonatal ICU, Emerg = emergency, Oncol = oncology, CTICU = cardiothoracic ICU, and N.S. = not stated.
* Percentage compliance before/after patient contact.
† After contact with inanimate objects.

Factors Affecting Adherence. Factors that may influence hand hygiene include those identified in epidemiologic studies and factors reported by HCWs as being reasons for lack of adherence to hand-hygiene recommendations. Risk factors for poor adherence to hand hygiene have been determined objectively in several observational studies or interventions to improve adherence (*11,12,274,292,295,314–317*). Among these, being a physician or a nursing assistant, rather than a nurse, was consistently associated with reduced adherence (Box 1).

In the largest hospitalwide survey of hand-hygiene practices among HCWs *(11)*, predictors of poor adherence to recommended hand-hygiene measures were identified. Predictor variables included professional category, hospital ward, time of day/week, and type and intensity of patient care, defined as the number of opportunities for hand hygiene per hour of patient care. In 2,834 observed opportunities for hand hygiene, average adherence was 48%. In multivariate analysis, nonadherence was lowest among nurses and during weekends

BOX 1. Factors influencing adherence to hand-hygiene practices*

Observed risk factors for poor adherence to recommended hand-hygiene practices

- Physician status (rather than a nurse)
- Nursing assistant status (rather than a nurse)
- Male sex
- Working in an intensive-care unit
- Working during the week (versus the weekend)
- Wearing gowns/gloves
- Automated sink
- Activities with high risk of cross-transmission
- High number of opportunities for hand hygiene per hour of patient care

Self-reported factors for poor adherence with hand hygiene

- Handwashing agents cause irritation and dryness
- Sinks are inconveniently located/shortage of sinks
- Lack of soap and paper towels
- Often too busy/insufficient time
- Understaffing/overcrowding
- Patient needs take priority
- Hand hygiene interferes with health-care worker relationships with patients
- Low risk of acquiring infection from patients
- Wearing of gloves/beliefs that glove use obviates the need for hand hygiene
- Lack of knowledge of guidelines/protocols
- Not thinking about it/forgetfulness
- No role model from colleagues or superiors
- Skepticism regarding the value of hand hygiene
- Disagreement with the recommendations
- Lack of scientific information of definitive impact of improved hand hygiene on health-care–associated infection rates

Additional perceived barriers to appropriate hand hygiene

- Lack of active participation in hand-hygiene promotion at individual or institutional level
- Lack of role model for hand hygiene
- Lack of institutional priority for hand hygiene
- Lack of administrative sanction of noncompliers/rewarding compliers
- Lack of institutional safety climate

* Source: Adapted from Pittet D. Improving compliance with hand hygiene in hospitals. Infect Control Hosp Epidemiol 2000;21:381–6.

(Odds Ratio [OR]: 0.6; 95% confidence interval [CI] = 0.4–0.8). Nonadherence was higher in intensive-care units compared with internal medicine wards (OR: 2.0; 95% CI = 1.3–3.1), during procedures that carried a high risk of bacterial contamination (OR: 1.8; 95% CI = 1.4–2.4), and when intensity of patient care was high (21–40 handwashing opportunities — OR: 1.3; 95% CI = 1.0-1.7; 41–60 opportunities — OR: 2.1; 95% CI = 1.5-2.9; >60 opportunities — OR: 2.1; 95% CI = 1.3–3.5). The higher the demand for hand hygiene, the lower the adherence; on average, adherence decreased by 5% (± 2%) for each increase of 10 opportunities per hour when the intensity of patient care exceeded 10 opportunities per hour. Similarly, the lowest adherence rate (36%) was found in intensive-care units, where indications for hand hygiene were typically more frequent (on average, 20 opportunities per patient-hour). The highest adherence rate (59%) was observed in pediatrics wards, where the average intensity of patient care was lower than in other hospital areas (an average of eight opportunities per patient-hour). The results of this study indicate that full adherence to previous guidelines may be unrealistic, and that facilitated access to hand hygiene could help improve adherence (*11,12,318*).

Perceived barriers to adherence with hand-hygiene practice recommendations include skin irritation caused by hand-hygiene agents, inaccessible hand-hygiene supplies, interference with HCW-patient relationships, priority of care (i.e., the patients' needs are given priority over hand hygiene), wearing of gloves, forgetfulness, lack of knowledge of the guidelines, insufficient time for hand hygiene, high workload and understaffing, and the lack of scientific information indicating a definitive impact of improved hand hygiene on health-care–associated infection rates (*11,274,292,295,315–317*). Certain perceived barriers to adherence with hand-hygiene guidelines have been assessed or quantified in observational studies (*12,274,292,295,314–317*) (Box 1).

Skin irritation by hand-hygiene agents constitutes a substantial barrier to appropriate adherence (*319*). Because soaps and detergents can damage skin when applied on a regular basis, HCWs must be better informed regarding the possible adverse effects associated with hand-hygiene agents. Lack of knowledge and education regarding this subject is a barrier to motivation. In several studies, alcohol-based hand rubs containing emollients (either isopropanol, ethanol, or n-propanol in 60%–90% vol/vol) were less irritating to the skin than the soaps or detergents tested. In addition, the alcohol-based products containing emollients that were tested were at least as tolerable and efficacious as the detergents tested. Also, studies demonstrate that several hand lotions have reduced skin scaling and cracking, which may reduce microbial shedding from the hands (*67,272,273*).

Easy access to hand-hygiene supplies, whether sink, soap, medicated detergent, or alcohol-based hand-rub solution, is essential for optimal adherence to hand-hygiene recommendations. The time required for nurses to leave a patient's bedside, go to a sink, and wash and dry their hands before attending the next patient is a deterrent to frequent handwashing or hand antisepsis (*11,318*). Engineering controls could facilitate adherence, but careful monitoring of hand-hygiene behavior should be conducted to exclude the possible negative effect of newly introduced handwashing devices (*88*).

The impact of wearing gloves on adherence to hand-hygiene policies has not been definitively established, because published studies have yielded contradictory results (*87,290,301,320*). Hand hygiene is required regardless of whether gloves are used or changed. Failure to remove gloves after patient contact or between "dirty" and "clean" body-site care on the same patient must be regarded as nonadherence to hand-hygiene recommendations (*11*). In a study in which experimental conditions approximated those occurring in clinical practice (*321*), washing and reusing gloves between patient contacts resulted in observed bacterial counts of 0–4.7 log on the hands after glove removal. Therefore, this practice should be discouraged; handwashing or disinfection should be performed after glove removal.

Lack of 1) knowledge of guidelines for hand hygiene, 2) recognition of hand-hygiene opportunities during patient care, and 3) awareness of the risk of cross-transmission of pathogens are barriers to good hand-hygiene practices. Furthermore, certain HCWs believe they have washed their hands when necessary, even when observations indicate they have not (*89,92,295,296,322*).

Perceived barriers to hand-hygiene behavior are linked not only to the institution, but also to HCWs' colleagues. Therefore, both institutional and small-group dynamics need to be considered when implementing a system change to secure an improvement in HCWs' hand-hygiene practice.

Possible Targets for Hand-Hygiene Promotion

Targets for the promotion of hand hygiene are derived from studies assessing risk factors for nonadherence, reported reasons for the lack of adherence to recommendations, and additional factors perceived as being important to facilitate appropriate HCW behavior. Although certain factors cannot be modified (Box 1), others can be changed.

One factor that must be addressed is the time required for HCWs to clean their hands. The time required for traditional handwashing may render full adherence to previous guidelines unrealistic (*11,12,318*) and more rapid access to hand-hygiene materials could help improve adherence. One study conducted in an intensive-care unit demonstrated that it took

nurses an average of 62 seconds to leave a patient's bedside, walk to a sink, wash their hands, and return to patient care (*318*). In contrast, an estimated one fourth as much time is required when using alcohol-based hand rub placed at each patient's bedside. Providing easy access to hand-hygiene materials is mandatory for appropriate hand-hygiene behavior and is achievable in the majority of health-care facilities (*323*). In particular, in high-demand situations (e.g., the majority of critical-care units), under hectic working conditions, and at times of overcrowding or understaffing, HCWs may be more likely to use an alcohol-based hand rub than to wash their hands (*323*). Further, using alcohol-based hand rubs may be a better option than traditional handwashing with plain soap and water or antiseptic handwash, because they not only require less time (*166,318*) but act faster (*1*) and irritate hands less often (*1,67,96,98,166*). They also were used in the only program that reported a sustained improvement in hand-hygiene adherence associated with decreased infection rates (*74*). However, making an alcohol-based hand rub available to personnel without providing ongoing educational and motivational activities may not result in long-lasting improvement in hand-hygiene practices (*313*). Because increased use of hand-hygiene agents might be associated with skin dryness, the availability of free skin-care lotion is recommended.

Education is a cornerstone for improvement with hand-hygiene practices. Topics that must be addressed by educational programs include the lack of 1) scientific information for the definitive impact of improved hand hygiene on health-care–associated infection and resistant organism transmission rates; 2) awareness of guidelines for hand hygiene and insufficient knowledge concerning indications for hand hygiene during daily patient care; 3) knowledge concerning the low average adherence rate to hand hygiene by the majority of HCWs; and 4) knowledge concerning the appropriateness, efficacy, and understanding of the use of hand-hygiene and skin-care–protection agents.

HCWs necessarily evolve within a group that functions within an institution. Possible targets for improvement in hand-hygiene behavior not only include factors linked to individual HCWs, but also those related to the group(s) and the institution as a whole (*317,323*). Examples of possible targets for hand-hygiene promotion at the group level include education and performance feedback on hand-hygiene adherence; efforts to prevent high workload, downsizing, and understaffing; and encouragement and provision of role models from key members in the work unit. At the institutional level, targets for improvement include 1) written guidelines, hand-hygiene agents, skin-care promotions and agents, or hand-hygiene facilities; 2) culture or tradition of adherence; and 3) administrative leadership, sanction, support, and rewards. Several studies, conducted in various types of institutions, reported modest and even low levels of adherence to recommended hand-hygiene practices, indicating that such adherence varied by hospital ward and by type of HCW. These results indicate educational sessions may need to be designed specifically for certain types of personnel (*11,289,290,294,317,323*).

Lessons Learned from Behavioral Theories

In 1998, the prevailing behavioral theories and their applications with regard to the health professions were reviewed by researchers in an attempt to better understand how to target more successful interventions (*317*). The researchers proposed a hypothetical framework to enhance hand-hygiene practices and stressed the importance of considering the complexity of individual and institutional factors when designing behavioral interventions.

Although behavioral theories and secondary interventions have primarily targeted individual workers, this practice might be insufficient to produce sustained change (*317,324,325*). Interventions aimed at improving hand-hygiene practices must account for different levels of behavior interaction (*12,317,326*). Thus, the interdependence of individual factors, environmental constraints, and the institutional climate must be taken into account in the strategic planning and development of hand-hygiene campaigns. Interventions to promote hand hygiene in hospitals should consider variables at all these levels. Various factors involved in hand-hygiene behavior include intention, attitude towards the behavior, perceived social norm, perceived behavioral control, perceived risk for infection, hand-hygiene practices, perceived role model, perceived knowledge, and motivation (*317*). The factors necessary for change include 1) dissatisfaction with the current situation, 2) perception of alternatives, and 3) recognition, both at the individual and institutional level, of the ability and potential to change. Although the latter implies education and motivation, the former two necessitate a system change.

Among the reported reasons for poor adherence with hand-hygiene recommendations (Box 1), certain ones are clearly associated with the institution or system (e.g., lack of institutional priority for hand hygiene, administrative sanctions, and a safety climate). Although all of these reasons would require a system change in the majority of institutions, the third requires management commitment, visible safety programs, an acceptable level of work stress, a tolerant and supportive attitude toward reported problems, and belief in the efficacy

of preventive strategies (*12,317,325,327*). Most importantly, an improvement in infection-control practices requires 1) questioning basic beliefs, 2) continuous assessment of the group (or individual) stage of behavioral change, 3) intervention(s) with an appropriate process of change, and 4) supporting individual and group creativity (*317*). Because of the complexity of the process of change, single interventions often fail. Thus, a multimodal, multidisciplinary strategy is likely necessary (*74,75,317,323,326*).

Methods Used To Promote Improved Hand Hygiene

Hand-hygiene promotion has been challenging for >150 years. In-service education, information leaflets, workshops and lectures, automated dispensers, and performance feedback on hand-hygiene adherence rates have been associated with transient improvement (*291,294–296,306,314*).

Several strategies for promotion of hand hygiene in hospitals have been published (Table 9). These strategies require education, motivation, or system change. Certain strategies are based on epidemiologic evidence, others on the authors' and other investigators' experience and review of current knowledge. Some strategies may be unnecessary in certain circumstances, but may be helpful in others. In particular, changing the hand-hygiene agent could be beneficial in institutions or hospital wards with a high workload and a high demand for hand hygiene when alcohol-based hand rubs are not available (*11,73,78,328*). However, a change in the recommended hand-hygiene agent could be deleterious if introduced during winter, at a time of higher hand-skin irritability, and if not accompanied by the provision of skin-care products (e.g., protective creams and lotions). Additional specific elements should be considered for inclusion in educational and motivational programs (Box 2).

Several strategies that could potentially be associated with successful promotion of hand hygiene require a system change (Box 1). Hand-hygiene adherence and promotion involve factors at both the individual and system level. Enhancing individual and institutional attitudes regarding the feasibility of making changes (self-efficacy), obtaining active participation of personnel at both levels, and promoting an institutional safety climate represent challenges that exceed the current perception of the role of infection-control professionals.

Whether increased education, individual reinforcement technique, appropriate rewarding, administrative sanction, enhanced self-participation, active involvement of a larger number of organizational leaders, enhanced perception of health threat, self-efficacy, and perceived social pressure (*12,317,329,330*), or combinations of these factors can improve HCWs' adherence with hand hygiene needs further investigation. Ultimately, adherence to recommended hand-hygiene practices should become part of a culture of patient safety where a set of interdependent quality elements interact to achieve a shared objective (*331*).

On the basis of both these hypothetical considerations and successful, actual experiences in certain institutions, strategies to improve adherence to hand-hygiene practices should be both multimodal and multidisciplinary. However, strategies must be further researched before they are implemented.

TABLE 9. Stategies for successful promotion of hand hygiene in hospitals

Strategy	Tool for change*	Selected references†
Education	E (M, S)	(*74,295,306,326,393*)
Routine observation and feedback	S (E, M)	(*74,294,306,326,393*)
Engineering control		
Make hand hygiene possible, easy, and convenient	S	(*74,281,326,393*)
Make alcohol-based hand rub available	S	(*74*)
(at least in high-demand situations)	S	(*74,283,312*)
Patient education	S (M)	(*283,394*)
Reminders in the workplace	S	(*74,395*)
Administrative sanction/rewarding	S	(*12,317*)
Change in hand-hygiene agent	S (E)	(*11,67,71,283,312*)
Promote/facilitate skin care for health-care–workers' hands	S (E)	(*67,74,274,275*)
Obtain active participation at individual and institutional level	E, M, S	(*74,75,317*)
Improve institutional safety climate	S (M)	(*74,75,317*)
Enhance individual and institutitional self-efficacy	S (E, M)	(*74,75,317*)
Avoid overcrowding, understaffing, and excessive workload	S	(*11,74,78,297,396*)
Combine several of above strategies	E, M, S	(*74,75,295,306,317,326*)

*The dynamic of behavioral change is complex and involves a combination of education (E), motivation (M), and system change (S).
†Only selected references have been listed; readers should refer to more extensive reviews for exhaustive reference lists (*1,8,317,323,397*).

BOX 2. Elements of health-care worker educational and motivational programs

Rationale for hand hygiene

- Potential risks of transmission of microorganisms to patients
- Potential risks of health-care worker colonization or infection caused by organisms acquired from the patient
- Morbidity, mortality, and costs associated with health-care–associated infections

Indications for hand hygiene

- Contact with a patient's intact skin (e.g., taking a pulse or blood pressure, performing physical examinations, lifting the patient in bed) (*25,26,45,48,51,53*)
- Contact with environmental surfaces in the immediate vicinity of patients (*46,51,53,54*)
- After glove removal (*50,58,71*)

Techniques for hand hygiene

- Amount of hand-hygiene solution
- Duration of hand-hygiene procedure
- Selection of hand-hygiene agents
  - — Alcohol-based hand rubs are the most efficacious agents for reducing the number of bacteria on the hands of personnel. Antiseptic soaps and detergents are the next most effective, and non-antimicrobial soaps are the least effective (*1,398*).
  - — Soap and water are recommended for visibly soil hands.
  - — Alcohol-based hand rubs are recommended for routine decontamination of hands for all clinical indications (except when hands are visibly soiled) and as one of the options for surgical hand hygiene.

Methods to maintain hand skin health

- Lotions and creams can prevent or minimize skin dryness and irritation caused by irritant contact dermatitis
- Acceptable lotions or creams to use
- Recommended schedule for applying lotions or creams

Expectations of patient care managers/administrators

- Written statements regarding the value of, and support for, adherence to recommended hand-hygiene practices
- Role models demonstrating adherence to recommended hand hygiene practices (*399*)

Indications for, and limitations of, glove use

- Hand contamination may occur as a result of small, undetected holes in examination gloves (*321,361*)
- Contamination may occur during glove removal (50)
- Wearing gloves does not replace the need for hand hygiene (58)
- Failure to remove gloves after caring for a patient may lead to transmission of microorganizations from one patient to another *(373).*

Efficacy of Promotion and Impact of Improved Hand Hygiene

The lack of scientific information of the definitive impact of improved hand hygiene on health-care–associated infection rates is a possible barrier to appropriate adherence with hand-hygiene recommendations (Box 1). However, evidence supports the belief that improved hand hygiene can reduce health-care–associated infection rates. Failure to perform appropriate hand hygiene is considered the leading cause of health-care–associated infections and spread of multiresistant organisms and has been recognized as a substantial contributor to outbreaks.

Of nine hospital-based studies of the impact of hand hygiene on the risk of health-care–associated infections (Table 10) (*48,69–75,296*), the majority demonstrated a temporal relationship between improved hand-hygiene practices and reduced infection rates.

In one of these studies, endemic MRSA in a neonatal intensive-care unit was eliminated 7 months after introduction of a new

TABLE 10. Association between improved adherence with hand-hygiene practice and health-care–associated infection rates

Year	Ref. no.	Hospital setting	Results	Duration of follow-up
1977	(*48*)	Adult ICU	Reduction in health-care–associated infections caused by endemic *Klebsiella* spp.	2 years
1982	(*69*)	Adult ICU	Reduction in health-care-associated infection rates	N.S.
1984	(*70*)	Adult ICU	Reduction in health-care–associated infection rates	N.S.
1990	(*296*)	Adult ICU	No effect (average hand hygiene adherence improvement did not reach statistical significance)	11 months
1992	(*71*)	Adult ICU	Substantial difference between rates of health-care–associated infection between two different hand-hygiene agents	8 months
1994	(*72*)	NICU	Elimination of MRSA, when combined with multiple other infection-control measures. Reduction of vancomycin use	9 months
1995	(*73*)	Newborn nursery	Elimination of MRSA, when combined with multiple other infection-control measures	3.5 years
2000	(*75*)	MICU/NICU	85% relative reduction of VRE rate in the intervention hospital; 44% relative reduction in control hospital; no change in MRSA	8 months
2000	(*74*)	Hospitalwide	Substantial reduction in the annual overall prevalence of health-care–associated infections and MRSA cross-transmission rates. Active surveillance cultures and contact precautions were implemented during same period	5 years

Note: ICU = intensive care unit, NICU = neonatal ICU, MRSA = methicillin-resistant *Staphylococcus aureus*, MICU = medical ICU, and N.S. = not stated.

hand antiseptic (1% triclosan); all other infection-control measures remained in place, including the practice of conducting weekly active surveillance by obtaining cultures (*72*). Another study reported an MRSA outbreak involving 22 infants in a neonatal unit (*73*). Despite intensive efforts, the outbreak could not be controlled until a new antiseptic was added (i.e., 0.3% triclosan); all previously used control measures remained in place, including gloves and gowns, cohorting, and obtaining cultures for active surveillance.

The effectiveness of a longstanding, hospitalwide program to promote hand hygiene at the University of Geneva hospitals was recently reported (*74*). Overall adherence to hand-hygiene guidelines during routine patient care was monitored during hospitalwide observational surveys. These surveys were conducted biannually during December 1994–December 1997, before and during implementation of a hand-hygiene campaign that specifically emphasized the practice of bedside, alcohol-based hand disinfection. Individual-sized bottles of hand-rub solution were distributed to all wards, and custom-made holders were mounted on all beds to facilitate access to hand disinfection. HCWs were also encouraged to carry bottles in their pockets, and in 1996, a newly designed flat (instead of round) bottle was made available to further facilitate pocket carriage. The promotional strategy was multimodal and involved a multidisciplinary team of HCWs, the use of wall posters, the promotion of antiseptic hand rubs located at bedsides throughout the institution, and regular performance feedback to all HCWs (see http://www.hopisafe.ch for further details on methodology). Health-care–associated infection rates, attack rates of MRSA cross-transmission, and consumption of hand-rub disinfectant were measured. Adherence to recommended hand-hygiene practices improved progressively from 48% in 1994 to 66% in 1997 ($p < 0.001$). Whereas recourse to handwashing with soap and water remained stable, frequency of hand disinfection markedly increased during the study period ($p < 0.001$), and the consumption of alcohol-based hand-rub solution increased from 3.5 to 15.4 liters per 1,000 patient-days during 1993–1998 ($p < 0.001$). The increased frequency of hand disinfection was unchanged after adjustment for known risk factors of poor adherence. During the same period, both overall health-care–associated infection and MRSA transmission rates decreased (both $p < 0.05$). The observed reduction in MRSA transmission may have been affected by both improved hand-hygiene adherence and the simultaneous implementation of active surveillance cultures for detecting and isolating patients colonized with MRSA (*332*). The experience from the University of Geneva hospitals constitutes the first report of a hand-hygiene campaign with a sustained improvement over several years. An additional multimodal program also yielded sustained improvements in hand-hygiene practices over an extended period (*75*); the majority of studies have been limited to a 6- to 9-month observation period.

Although these studies were not designed to assess the independent contribution of hand hygiene on the prevention of health-care–associated infections, the results indicate that

improved hand-hygiene practices reduce the risk of transmission of pathogenic microorganisms. The beneficial effects of hand-hygiene promotion on the risk of cross-transmission also have been reported in surveys conducted in schools and day care centers (*333–338*), as well as in a community setting (*339–341*).

Other Policies Related to Hand Hygiene

Fingernails and Artificial Nails

Studies have documented that subungual areas of the hand harbor high concentrations of bacteria, most frequently coagulase-negative staphylococci, gram-negative rods (including *Pseudomonas* spp.), Corynebacteria, and yeasts (*14,342,343*). Freshly applied nail polish does not increase the number of bacteria recovered from periungual skin, but chipped nail polish may support the growth of larger numbers of organisms on fingernails (*344,345*). Even after careful handwashing or the use of surgical scrubs, personnel often harbor substantial numbers of potential pathogens in the subungual spaces (*346–348*).

Whether artificial nails contribute to transmission of health-care–associated infections is unknown. However, HCWs who wear artificial nails are more likely to harbor gram-negative pathogens on their fingertips than are those who have natural nails, both before and after handwashing (*347–349*). Whether the length of natural or artificial nails is a substantial risk factor is unknown, because the majority of bacterial growth occurs along the proximal 1 mm of the nail adjacent to subungual skin (*345,347,348*). Recently, an outbreak of *P. aeruginosa* in a neonatal intensive care unit was attributed to two nurses (one with long natural nails and one with long artificial nails) who carried the implicated strains of *Pseudomonas* spp. on their hands (*350*). Patients were substantially more likely than controls to have been cared for by the two nurses during the exposure period, indicating that colonization of long or artificial nails with *Pseudomonas* spp. may have contributed to causing the outbreak. Personnel wearing artificial nails also have been epidemiologically implicated in several other outbreaks of infection caused by gram-negative bacilli and yeast (*351–353*). Although these studies provide evidence that wearing artificial nails poses an infection hazard, additional studies are warranted.

Gloving Policies

CDC has recommended that HCWs wear gloves to 1) reduce the risk of personnel acquiring infections from patients, 2) prevent health-care worker flora from being transmitted to patients, and 3) reduce transient contamination of the hands of personnel by flora that can be transmitted from one patient to another (*354*). Before the emergence of the acquired immunodeficiency syndrome (AIDS) epidemic, gloves were worn primarily by personnel caring for patients colonized or infected with certain pathogens or by personnel exposed to patients with a high risk of hepatitis B. Since 1987, a dramatic increase in glove use has occurred in an effort to prevent transmission of HIV and other bloodborne pathogens from patients to HCWs (*355*). The Occupational Safety and Health Administration (OSHA) mandates that gloves be worn during all patient-care activities that may involve exposure to blood or body fluids that may be contaminated with blood (*356*).

The effectiveness of gloves in preventing contamination of HCWs' hands has been confirmed in several clinical studies (*45,51,58*). One study found that HCWs who wore gloves during patient contact contaminated their hands with an average of only 3 CFUs per minute of patient care, compared with 16 CFUs per minute for those not wearing gloves (*51*). Two other studies, involving personnel caring for patients with *C. difficile* or VRE, revealed that wearing gloves prevented hand contamination among the majority of personnel having direct contact with patients (*45,58*). Wearing gloves also prevented personnel from acquiring VRE on their hands when touching contaminated environmental surfaces (*58*). Preventing heavy contamination of the hands is considered important, because handwashing or hand antisepsis may not remove all potential pathogens when hands are heavily contaminated (*25,111*).

Several studies provide evidence that wearing gloves can help reduce transmission of pathogens in health-care settings. In a prospective controlled trial that required personnel to routinely wear vinyl gloves when handling any body substances, the incidence of *C. difficile* diarrhea among patients decreased from 7.7 cases/1,000 patient discharges before the intervention to 1.5 cases/1,000 discharges during the intervention (*226*). The prevalence of asymptomatic *C. difficile* carriage also decreased substantially on "glove" wards, but not on control wards. In intensive-care units where VRE or MRSA have been epidemic, requiring all HCWs to wear gloves to care for all patients in the unit (i.e., universal glove use) likely has helped control outbreaks (*357,358*).

The influence of glove use on the hand-hygiene habits of personnel is not clear. Several studies found that personnel who wore gloves were less likely to wash their hands upon leaving a patient's room (*290,320*). In contrast, two other studies found that personnel who wore gloves were substantially more likely to wash their hands after patient care (*87,301*).

The following caveats regarding use of gloves by HCWs must be considered. Personnel should be informed that gloves

do not provide complete protection against hand contamination. Bacterial flora colonizing patients may be recovered from the hands of ≤30% of HCWs who wear gloves during patient contact (*50,58*). Further, wearing gloves does not provide complete protection against acquisition of infections caused by hepatitis B virus and herpes simplex virus (*359,360*). In such instances, pathogens presumably gain access to the caregiver's hands via small defects in gloves or by contamination of the hands during glove removal (*50,321,359,361*).

Gloves used by HCWs are usually made of natural rubber latex and synthetic nonlatex materials (e.g., vinyl, nitrile, and neoprene [polymers and copolymers of chloroprene]). Because of the increasing prevalence of latex sensitivity among HCWs and patients, FDA has approved several powdered and powder-free latex gloves with reduced protein contents, as well as synthetic gloves that can be made available by health-care institutions for use by latex-sensitive employees. In published studies, the barrier integrity of gloves varies on the basis of type and quality of glove material, intensity of use, length of time used, manufacturer, whether gloves were tested before or after use, and method used to detect glove leaks (*359,361–366*). In published studies, vinyl gloves have had defects more frequently than latex gloves, the difference in defect frequency being greatest after use (*359,361,364,367*). However, intact vinyl gloves provide protection comparable to that of latex gloves (*359*). Limited studies indicate that nitrile gloves have leakage rates that approximate those of latex gloves (*368–371*). Having more than one type of glove available is desirable, because it allows personnel to select the type that best suits their patient-care activities. Although recent studies indicate that improvements have been made in the quality of gloves (*366*), hands should be decontaminated or washed after removing gloves (*8,50,58,321,361*). Gloves should not be washed or reused (*321,361*). Use of petroleum-based hand lotions or creams may adversely affect the integrity of latex gloves (*372*). After use of powdered gloves, certain alcohol hand rubs may interact with residual powder on the hands of personnel, resulting in a gritty feeling on the hands. In facilities where powdered gloves are commonly used, various alcohol-based hand rubs should be tested after removal of powdered gloves to avoid selecting a product that causes this undesirable reaction. Personnel should be reminded that failure to remove gloves between patients may contribute to transmission of organisms (*358,373*).

Jewelry

Several studies have demonstrated that skin underneath rings is more heavily colonized than comparable areas of skin on fingers without rings (*374–376*). One study found that 40% of nurses harbored gram-negative bacilli (e.g., *E. cloacae*, *Klebsiella*, and *Acinetobacter*) on skin under rings and that certain nurses carried the same organism under their rings for several months (*375*). In a more recent study involving >60 intensive care unit nurses, multivariable analysis revealed that rings were the only substantial risk factor for carriage of gram-negative bacilli and *S. aureus* and that the concentration of organisms recovered correlated with the number of rings worn (*377*). Whether the wearing of rings results in greater transmission of pathogens is unknown. Two studies determined that mean bacterial colony counts on hands after handwashing were similar among persons wearing rings and those not wearing rings (*376,378*). Further studies are needed to establish if wearing rings results in greater transmission of pathogens in health-care settings.

Hand-Hygiene Research Agenda

Although the number of published studies concerning hand hygiene has increased considerably in recent years, many questions regarding hand-hygiene products and strategies for improving adherence of personnel to recommended policies remain unanswered. Several concerns must still be addressed by researchers in industry and by clinical investigators (Box 3).

Web-Based Hand-Hygiene Resources

Additional information regarding improving hand hygiene is available at http://www.hopisafe.ch
University of Geneva Hospitals, Geneva, Switzerland
http://www.cdc.gov/ncidod/hip
CDC, Atlanta, Georgia
http://www.jr2.ox.ac.uk/bandolier/band88/b88-8.html
Bandolier journal, United Kingdom
http://www.med.upenn.edu
University of Pennsylvania, Philadelphia, Pennsylvania

BOX 3. Hand-hygiene research agenda

Education and promotion

- Provide health-care workers (HCWs) with better education regarding the types of patient care activities that can result in hand contamination and cross-transmission of microorganisms.
- Develop and implement promotion hand-hygiene programs in pregraduate courses.
- Study the impact of population-based education on hand-hygiene behavior.
- Design and conduct studies to determine if frequent glove use should be encouraged or discouraged.
- Determine evidence-based indications for hand cleansing (considering that it might be unrealistic to expect HCWs to clean their hands after every contact with the patient).
- Assess the key determinants of hand-hygiene behavior and promotion among the different populations of HCWs.
- Develop methods to obtain management support.
- Implement and evaluate the impact of the different components of multimodal programs to promote hand hygiene.

Hand-hygiene agents and hand care

- Determine the most suitable formulations for hand-hygiene products.
- Determine if preparations with persistent antimicrobial activity reduce infection rates more effectively than do preparations whose activity is limited to an immediate effect.
- Study the systematic replacement of conventional handwashing by the use of hand disinfection.
- Develop devices to facilitate the use and optimal application of hand-hygiene agents.
- Develop hand-hygiene agents with low irritancy potential.
- Study the possible advantages and eventual interaction of hand-care lotions, creams, and other barriers to help minimize the potential irritation associated with hand-hygiene agents.

Laboratory-based and epidemiologic research and development

- Develop experimental models for the study of cross-contamination from patient to patient and from environment to patient.
- Develop new protocols for evaluating the in vivo efficacy of agents, considering in particular short application times and volumes that reflect actual use in health-care facilities.
- Monitor hand-hygiene adherence by using new devices or adequate surrogate markers, allowing frequent individual feedback on performance.
- Determine the percentage increase in hand-hygiene adherence required to achieve a predictable risk reduction in infection rates.
- Generate more definitive evidence for the impact on infection rates of improved adherence to recommended hand-hygiene practices.
- Provide cost-effectiveness evaluation of successful and unsuccessful promotion campaigns.

Part II. Recommendations

Categories

These recommendations are designed to improve hand-hygiene practices of HCWs and to reduce transmission of pathogenic microorganisms to patients and personnel in health-care settings. This guideline and its recommendations are not intended for use in food processing or food-service establishments, and are not meant to replace guidance provided by FDA's Model Food Code.

As in previous CDC/HICPAC guidelines, each recommendation is categorized on the basis of existing scientific data, theoretical rationale, applicability, and economic impact. The CDC/HICPAC system for categorizing recommendations is as follows:

Category IA. Strongly recommended for implementation and strongly supported by well-designed experimental, clinical, or epidemiologic studies.

Category IB. Strongly recommended for implementation and supported by certain experimental, clinical, or epidemiologic studies and a strong theoretical rationale.

Category IC. Required for implementation, as mandated by federal or state regulation or standard.

Category II. Suggested for implementation and supported by suggestive clinical or epidemiologic studies or a theoretical rationale.

No recommendation. Unresolved issue. Practices for which insufficient evidence or no consensus regarding efficacy exist.

Recommendations

1. Indications for handwashing and hand antisepsis
   - A. When hands are visibly dirty or contaminated with proteinaceous material or are visibly soiled with blood or other body fluids, wash hands with either a non-antimicrobial soap and water or an antimicrobial soap and water (IA) (*66*).
   - B. If hands are not visibly soiled, use an alcohol-based hand rub for routinely decontaminating hands in all other clinical situations described in items 1C–J (IA) (*74,93,166,169,283,294,312,398*). Alternatively, wash hands with an antimicrobial soap and water in all clinical situations described in items 1C–J (IB) (*69-71,74*).
   - C. Decontaminate hands before having direct contact with patients (IB) (*68,400*).
   - D. Decontaminate hands before donning sterile gloves when inserting a central intravascular catheter (IB) (*401,402*).
   - E. Decontaminate hands before inserting indwelling urinary catheters, peripheral vascular catheters, or other invasive devices that do not require a surgical procedure (IB) (*25,403*).
   - F. Decontaminate hands after contact with a patient's intact skin (e.g., when taking a pulse or blood pressure, and lifting a patient) (IB) (*25,45,48,68*).
   - G. Decontaminate hands after contact with body fluids or excretions, mucous membranes, nonintact skin, and wound dressings if hands are not visibly soiled (IA) (*400*).
   - H. Decontaminate hands if moving from a contaminated-body site to a clean-body site during patient care (II) (*25,53*).
   - I. Decontaminate hands after contact with inanimate objects (including medical equipment) in the immediate vicinity of the patient (II) (*46,53,54*).
   - J. Decontaminate hands after removing gloves (IB) (*50,58,321*).
   - K. Before eating and after using a restroom, wash hands with a non-antimicrobial soap and water or with an antimicrobial soap and water (IB) (*404-409*).
   - L. Antimicrobial-impregnated wipes (i.e., towelettes) may be considered as an alternative to washing hands with non-antimicrobial soap and water. Because they are not as effective as alcohol-based hand rubs or washing hands with an antimicrobial soap and water for reducing bacterial counts on the hands of HCWs, they are not a substitute for using an alcohol-based hand rub or antimicrobial soap (IB) (*160,161*).
   - M. Wash hands with non-antimicrobial soap and water or with antimicrobial soap and water if exposure to *Bacillus anthracis* is suspected or proven. The physical action of washing and rinsing hands under such circumstances is recommended because alcohols, chlorhexidine, iodophors, and other antiseptic agents have poor activity against spores (II) (*120,172, 224,225*).
   - N. No recommendation can be made regarding the routine use of nonalcohol-based hand rubs for hand hygiene in health-care settings. Unresolved issue.
2. Hand-hygiene technique
   - A. When decontaminating hands with an alcohol-based hand rub, apply product to palm of one hand and rub hands together, covering all surfaces of hands and fingers, until hands are dry (IB) (*288,410*). Follow the manufacturer's recommendations regarding the volume of product to use.
   - B. When washing hands with soap and water, wet hands first with water, apply an amount of product recommended by the manufacturer to hands, and rub hands together vigorously for at least 15 seconds, covering all surfaces of the hands and fingers. Rinse hands with water and dry thoroughly with a disposable towel. Use towel to turn off the faucet (IB) (*90-92,94,411*). Avoid using hot water, because repeated exposure to hot water may increase the risk of dermatitis (IB) (*254,255*).
   - C. Liquid, bar, leaflet or powdered forms of plain soap are acceptable when washing hands with a non-antimicrobial soap and water. When bar soap is used, soap racks that facilitate drainage and small bars of soap should be used (II) (*412-415*).
   - D. Multiple-use cloth towels of the hanging or roll type are not recommended for use in health-care settings (II) (*137,300*).
3. Surgical hand antisepsis
   - A. Remove rings, watches, and bracelets before beginning the surgical hand scrub (II) (*375,378,416*).
   - B. Remove debris from underneath fingernails using a nail cleaner under running water (II) (*14,417*).

C. Surgical hand antisepsis using either an antimicrobial soap or an alcohol-based hand rub with persistent activity is recommended before donning sterile gloves when performing surgical procedures (IB) (*115,159,232,234,237,418*).

D. When performing surgical hand antisepsis using an antimicrobial soap, scrub hands and forearms for the length of time recommended by the manufacturer, usually 2–6 minutes. Long scrub times (e.g., 10 minutes) are not necessary (IB) (*117,156,205, 207,238-241*).

E. When using an alcohol-based surgical hand-scrub product with persistent activity, follow the manufacturer's instructions. Before applying the alcohol solution, prewash hands and forearms with a non-antimicrobial soap and dry hands and forearms completely. After application of the alcohol-based product as recommended, allow hands and forearms to dry thoroughly before donning sterile gloves (IB) (*159,237*).

4. Selection of hand-hygiene agents

A. Provide personnel with efficacious hand-hygiene products that have low irritancy potential, particularly when these products are used multiple times per shift (IB) (*90,92,98,166,249*). This recommendation applies to products used for hand antisepsis before and after patient care in clinical areas and to products used for surgical hand antisepsis by surgical personnel.

B. To maximize acceptance of hand-hygiene products by HCWs, solicit input from these employees regarding the feel, fragrance, and skin tolerance of any products under consideration. The cost of hand-hygiene products should not be the primary factor influencing product selection (IB) (*92,93,166, 274,276-278*).

C. When selecting non-antimicrobial soaps, antimicrobial soaps, or alcohol-based hand rubs, solicit information from manufacturers regarding any known interactions between products used to clean hands, skin care products, and the types of gloves used in the institution (II) (*174,372*).

D. Before making purchasing decisions, evaluate the dispenser systems of various product manufacturers or distributors to ensure that dispensers function adequately and deliver an appropriate volume of product (II) (*286*).

E. Do not add soap to a partially empty soap dispenser. This practice of "topping off" dispensers can lead to bacterial contamination of soap (IA) (*187,419*).

5. Skin care

A. Provide HCWs with hand lotions or creams to minimize the occurrence of irritant contact dermatitis associated with hand antisepsis or handwashing (IA) (*272,273*).

B. Solicit information from manufacturers regarding any effects that hand lotions, creams, or alcohol-based hand antiseptics may have on the persistent effects of antimicrobial soaps being used in the institution (IB) (*174,420,421*).

6. Other Aspects of Hand Hygiene

A. Do not wear artificial fingernails or extenders when having direct contact with patients at high risk (e.g., those in intensive-care units or operating rooms) (IA) (*350–353*).

B. Keep natural nails tips less than 1/4-inch long (II) (*350*).

C. Wear gloves when contact with blood or other potentially infectious materials, mucous membranes, and nonintact skin could occur (IC) (*356*).

D. Remove gloves after caring for a patient. Do not wear the same pair of gloves for the care of more than one patient, and do not wash gloves between uses with different patients (IB) (*50,58,321,373*).

E. Change gloves during patient care if moving from a contaminated body site to a clean body site (II) (*50,51,58*).

F. No recommendation can be made regarding wearing rings in health-care settings. Unresolved issue.

7. Health-care worker educational and motivational programs

A. As part of an overall program to improve hand-hygiene practices of HCWs, educate personnel regarding the types of patient-care activities that can result in hand contamination and the advantages and disadvantages of various methods used to clean their hands (II) (*74,292,295,299*).

B. Monitor HCWs' adherence with recommended hand-hygiene practices and provide personnel with information regarding their performance (IA) (*74,276,292,295,299,306,310*).

C. Encourage patients and their families to remind HCWs to decontaminate their hands (II) (*394,422*).

8. Administrative measures

A. Make improved hand-hygiene adherence an institutional priority and provide appropriate

administrative support and financial resources (IB) (*74,75*).

B. Implement a multidisciplinary program designed to improve adherence of health personnel to recommended hand-hygiene practices (IB) (*74,75*).

C. As part of a multidisciplinary program to improve hand-hygiene adherence, provide HCWs with a readily accessible alcohol-based hand-rub product (IA) (*74,166,283,294,312*).

D. To improve hand-hygiene adherence among personnel who work in areas in which high workloads and high intensity of patient care are anticipated, make an alcohol-based hand rub available at the entrance to the patient's room or at the bedside, in other convenient locations, and in individual pocket-sized containers to be carried by HCWs (IA) (*11,74,166,283,284,312,318,423*).

E. Store supplies of alcohol-based hand rubs in cabinets or areas approved for flammable materials (IC).

Part III. Performance Indicators

1. The following performance indicators are recommended for measuring improvements in HCWs' hand-hygiene adherence:
   A. Periodically monitor and record adherence as the number of hand-hygiene episodes performed by personnel/number of hand-hygiene opportunities, by ward or by service. Provide feedback to personnel regarding their performance.
   B. Monitor the volume of alcohol-based hand rub (or detergent used for handwashing or hand antisepsis) used per 1,000 patient-days.
   C. Monitor adherence to policies dealing with wearing of artificial nails.
   D. When outbreaks of infection occur, assess the adequacy of health-care worker hand hygiene.

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Appendix

Antimicrobial Spectrum and Characteristics of Hand-Hygiene Antiseptic Agents*

Group	Gram-positive bacteria	Gram-negative bacteria	Mycobacteria	Fungi	Viruses	Speed of action	Comments
Alcohols	+++	+++	+++	+++	+++	Fast	Optimum concentration 60%–95%; no persistent activity
Chlorhexidine (2% and 4% aqueous)	+++	++	+	+	+++	Intermediate	Persistent activity; rare allergic reactions
Iodine compounds	+++	+++	+++	++	+++	Intermediate	Causes skin burns; usually too irritating for hand hygiene
Iodophors	+++	+++	+	++	++	Intermediate	Less irritating than iodine; acceptance varies
Phenol derivatives	+++	+	+	+	+	Intermediate	Activity neutralized by nonionic surfactants
Tricolsan	+++	++	+	—	+++	Intermediate	Acceptability on hands varies
Quaternary ammonium compounds	+	++	—	—	+	Slow	Used only in combination with alcohols; ecologic concerns

Note: +++ = excellent; ++ = good, but does not include the entire bacterial spectrum; + = fair; — = no activity or not sufficient.
* Hexachlorophene is not included because it is no longer an accepted ingredient of hand disinfectants.

MMWR™

Morbidity and Mortality Weekly Report

Recommendations and Reports — **October 25, 2002 / Vol. 51 / No. RR-16**

Continuing Education Activity Sponsored by CDC
Guideline for Hand Hygiene in Health-Care Settings

Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force

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Goal and Objectives

This *MMWR* provides evidence-based recommendations for hand hygiene in health-care settings. These recommendations were developed by the Healthcare Infection Control Practices Advisory Committee (HICPAC), the Society for Healthcare Epidemiology of America, the Association for Professionals in Infection Control and Epidemiology, and the Infectious Diseases Society of America Hand Hygiene Task Force. The goal of this report is to provide guidance for clinicians and other health-care practitioners regarding strategies to improve hand-hygiene practices and reduce transmission of microorganisms in health-care settings. Upon completion of this educational activity, the reader should be able to 1) describe the indications for hand hygiene in health-care settings; 2) list the advantages of alcohol-based hand rubs; and 3) describe the barriers to hand hygiene in health-care settings.

To receive continuing education credit, please answer all of the following questions.

1. **Hand hygiene refers to . . .**
   - A. handwashing using plain soap and water.
   - B. using an antiseptic hand rub (e.g alcohol, chlorhexidine, iodine).
   - C. handwashing using antimicrobial soap and water.
   - D. all of the above.

2. **Hand hygiene adherence in health-care facilities might be improved by . . .**
   - A. providing personnel with individual containers of alcohol-based hand rubs.
   - B. providing personnel with hand lotions or creams.
   - C. providing personnel with feedback regarding hand-hygiene adherence/performance.
   - D. all of the above.

3. **Alcohol-based hand rubs have good or excellent antimicrobial activity against all of the following except . . .**
   - A. viruses.
   - B. fungi.
   - C. mycobacteria.
   - D. bacterial spores.
   - E. gram-positive and gram-negative bacteria.

4. **Alcohol-based hand rubs are indicated for all of the following clinical situations except . . .**
   - A. when the hands are visibly soiled.
   - B. preoperative cleaning of hands by surgical personnel.
   - C. before inserting urinary catheters, intravascular catheters, or other invasive devices.
   - D. after removing gloves.

5. **Each of the following statements regarding alcohol-based hand rubs is true except . . .**
   - A. alcohol-based hand rubs reduce bacterial counts on the hands of health-care personnel more effectively than plain soaps.
   - B. alcohol-based hand rubs can be made more accessible than sinks or other handwashing facilities.
   - C. alcohol-based hand rubs require less time to use than traditional handwashing.
   - D. alcohol-based hand rubs have been demonstrated to cause less skin irritation and dryness than handwashing using soap and water.
   - E. alcohol-based hand rubs are only effective if they are applied for ≥60 seconds.

6. **Which of the following statements regarding preoperative surgical hand antisepsis is true?**
   - A. Antimicrobial counts on hands are reduced as effectively with a 5-minute scrub as with a 10-minute scrub.
   - B. A brush or sponge must be used when applying the antiseptic agent to adequately reduce bacterial counts on hands.
   - C. Alcohol-based hand rubs for preoperative surgical scrub have been associated with increased surgical site infection rates.
   - D. A and B are true.
   - E. A and C are true.

7. **Antimicrobial-impregnated wipes (i.e., towelettes) . . .**
   - A. might be considered as an alternative to handwashing with plain soap and water.
   - B. are as effective as alcohol-based hands rubs.
   - C. are as effective as washing hands with antimicrobial soap and water.
   - D. A and C.

8. **The following statements regarding hand hygiene in health-care settings are true except . . .**
   - A. Overall adherence among health-care personnel is approximately 40%.
   - B. Poor adherence to hand-hygiene practice is a primary contributor to health-care–associated infection and transmission of antimicrobial-resistant pathogens.
   - C. Personnel wearing artificial nails or extenders have been linked to nosocomial outbreaks.
   - D. Hand hygiene is not necessary if gloves are worn.

9. **Indicate your work setting.**
   - A. State/local health department.
   - B. Other public health setting.
   - C. Hospital clinic/private practice.
   - D. Managed care organization.
   - E. Academic institution.
   - F. Other.

10. **Which best describes your professional activities?**
    - A. Patient care — emergency/urgent care department.
    - B. Patient care — inpatient.
    - C. Patient care — primary-care clinic or office.
    - D. Laboratory/pharmacy.
    - E. Public health.
    - F. Other.

11. **I plan to use these recommendations as the basis for . . . (*Indicate all that apply.*)**
    - A. health education materials.
    - B. insurance reimbursement policies.
    - C. local practice guidelines.
    - D. public policy.
    - E. other.

12. **Each month, approximately how many patients do you examine?**
    - A. None.
    - B. 1–5.
    - C. 6–20.
    - D. 21–50.
    - E. 51–100.
    - F. >100.

13. **How much time did you spend reading this report and completing the exam?**
    - A. 1–1.5 hours.
    - B. More than 1.5 hours but fewer than 2 hours.
    - C. 2–2.5 hours.
    - D. More than 2.5 hours.

14. **After reading this report, I am confident I can describe the guidance for clinicians and other health-care practitioners regarding strategies to improve hand-hygiene practices and reduce transmission of microorganisms in health-care settings.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

15. **After reading this report, I am confident I can describe the indications for hand hygiene in health-care settings.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

16. **After reading this report, I am confident I can list the advantages of alcohol-based hand rubs.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

17. **After reading this report, I am confident I can describe the barriers to hand hygiene in health-care settings.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

18. **The objectives are relevant to the goal of this report.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

19. **The tables and text boxes are useful.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

20. **Overall, the presentation of the report enhanced my ability to understand the material.**
    A. Strongly agree.
    B. Agree.
    C. Neither agree nor disagree.
    D. Disagree.
    E. Strongly disagree.

Detach or photocopy.

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October 25, 2002/Vol. 51/No. RR-16
Guideline for Hand Hygiene in Health-Care Settings
Recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force

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5. [ ]A [ ]B [ ]C [ ]D [ ]E	18. [ ]A [ ]B [ ]C [ ]D [ ]E	
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9. [ ]A [ ]B [ ]C [ ]D [ ]E [ ]F	22. [ ]A [ ]B [ ]C [ ]D [ ]E	
10. [ ]A [ ]B [ ]C [ ]D [ ]E [ ]F	23. [ ]A [ ]B [ ]C [ ]D [ ]E [ ]F	
11. [ ]A [ ]B [ ]C [ ]D [ ]E		
12. [ ]A [ ]B [ ]C [ ]D [ ]E [ ]F		
13. [ ]A [ ]B [ ]C [ ]D		

Signature Date / Completed Exam

21. These recommendations will affect my practice.

A. Strongly agree.
B. Agree.
C. Neither agree nor disagree.
D. Disagree.
E. Strongly disagree.

22. The availability of continuing education credit influenced my decision to read this report.

A. Strongly agree.
B. Agree.
C. Neither agree nor disagree.
D. Disagree.
E. Strongly disagree.

23. How did you learn about this continuing education activity?

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D. Conference presentation.
E. *MMWR* subscription.
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Correct answers for questions 1–8
1. D; 2. D; 3. D; 4. A; 5. E; 6. A; 7. A; 8. D.

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Exhibit J

Submitted as Exhibit to Amendment of January 28, 2009

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Virucidal hand treatments for prevention of rhinovirus infection

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Rhinovirus infections are associated with substantial morbidity and economic cost. The available common cold remedies are of limited utility and specific antiviral approaches have been unsuccessful. Viral contamination of the hands appears to play an important role in the transmission of rhinovirus from person-to-person. Interruption of this step in transmission presents a potential target for intervention. Initial studies demonstrated that the common cold could be prevented by treatment of hands with iodine. Inactivation of the rhinoviruses by acid is well known and a survey of organic acids considered safe for consumer use revealed that salicylic acid and pyroglutamic acid have potent virucidal activity for the rhinoviruses that persists for several hours after application to the hands. A subsequent evaluation in human volunteers confirmed the prevention of rhinovirus infections by these acids and suggested that these agents have promise as cosmetically acceptable virucidal agents for interruption of the transmission of these infections.

Keywords: common cold, hand disinfection, antiviral

Introduction

Rhinovirus infections are the most frequent cause of common cold illnesses. These upper respiratory infections are generally mild and self-limited in the normal host, but they are associated with an enormous economic burden both in lost productivity and in expenditures for treatment. In addition, rhinovirus infection is frequently associated with medical complications that have substantial morbidity. Rhinovirus infection has been detected in up to 30% of children who seek medical care for acute otitis media and also appears to exacerbate underlying lower respiratory tract illnesses. Rhinovirus infection may also be associated with substantial morbidity in the elderly. Prevention or treatment of rhinovirus infections with resulting avoidance of these complications would have an enormous societal impact with regard to both medical morbidity and economic cost.

Treatment of rhinovirus infections

Treatment options for rhinovirus colds are unsatisfactory. The available common cold remedies have only modest effects on specific symptoms and have no effect on the development of otitis media or exacerbations of lower respiratory tract disease. There are ongoing attempts to develop antiviral treatments for rhinovirus colds. Substantial challenges remain, however, and it is not clear that this approach will ultimately result in effective treatment (reviewed in ref. 1). Pleconaril, an antiviral with good activity against the rhinoviruses, had only modest effects on the symptoms of the common cold in healthy adults.[2] The effect of pleconaril on the morbidity associated with rhinovirus infections in high-risk patients has not been carefully evaluated. An alternative to antiviral treatment—prevention of rhinovirus infection by interruption of person-to-person transmission—appears to be technologically and economically feasible.

Person-to-person transmission of rhinovirus

During a rhinovirus cold, virus replicates primarily in the epithelium of the nasal cavity and posterior nasopharynx. Potential mechanisms of spread of virus from the upper respiratory tract to a susceptible contact include small particle aerosols, large particle aerosols and contact spread either directly or via a fomite. Spread by direct contact implies that the infected individual contaminates themselves and/or the environment with rhinovirus that can be transferred to a susceptible 'recipient' by casual contact. The recipient then inoculates the virus onto the nasal mucosa by self-inoculation. The steps in these different modes of person-to-person transmission of rhinovirus infection have been studied in some detail. Aerosols generated by coughs and sneezes infrequently contain infectious virus. Virus was recovered in 1/13 sneezes and 0/8 coughs generated by adults with natural rhinovirus infections.[3] In contrast, virus can be recovered from the hands of ~40% of adults with rhinovirus colds.[3,4] The quantity of virus recovered from the hands is also generally greater than that recovered in coughs and sneezes and virus can readily be transferred to the hands of a 'recipient' by direct contact.[3,5–7]

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Transmission of rhinovirus by contact

Early observations suggesting that hand-to-hand contact was an important mode of transmission of rhinovirus prompted a search for compounds that would inactivate the virus on the hands. Of the disinfectants evaluated, only aqueous iodine was found to reliably eliminate virus on human skin.[8,9] Ethanol (70%) and combinations of ethanol with benzalkonium chloride or hexachlorophene were less active. A study in human volunteers demonstrated that 2% aqueous iodine could prevent the transmission of rhinovirus by direct contact.[7] The effect of hand treatment with 2% iodine on transmission of rhinovirus infection and common colds was then evaluated in the natural setting.[10] For this evaluation, the mothers in 206 families with at least one child were randomized to use either iodine or placebo hand treatment whenever respiratory symptoms appeared in another family member. The secondary attack rate of rhinovirus infection and common cold illness was then determined for mothers in the two treatment groups. Common cold illnesses developed after 16 (20%) of 79 exposures in the mothers in the control group for an attack rate of 40 illnesses/1000 days of exposure. In contrast, the iodine-treated mothers developed illness after four (7%) of 58 exposures for an attack rate of 13 illnesses/1000 days of exposure ($P = 0.05$). The attack rate for rhinovirus infection after exposure to a rhinovirus-positive contact was also examined in the two treatment groups. Rhinovirus transmission occurred in five (31%) of the 16 exposures in the control group compared with none of the 11 exposures in the iodine treatment group ($P = 0.06$). Although the power of this study was reduced by the insensitivity of standard cell culture isolation and serological techniques for detection of rhinovirus infections, the results support the concept that direct contact transmission of rhinovirus is an important mode of transmission in the natural setting and document that common cold illnesses can be prevented by the use of an effective virucidal hand treatment. Iodine is not acceptable for general use as a virucidal hand treatment because it discolours and dries the skin.

Development of virucidal hand treatments

The inactivation of rhinovirus by acids is well known and has been used for many years to distinguish the rhinoviruses from other picornaviruses. The mechanism of this inactivation appears to involve changes in the virion structure that result in loss of VP4 with an associated loss of infectivity.[11] Acid sensitivity has been used as the basis for attempts to develop effective virucidal agents against the rhinovirus.

Effective prevention of rhinovirus infection appears to require complete eradication of the virus from the hands. Previous studies have demonstrated that the 'human infectious dose' of rhinovirus for a susceptible individual is less than one tissue culture infectious dose.[12] Glutaric acid, evaluated as a potential virucidal agent in the 1980s, reduced viral titre but did not reliably eradicate virus from the hands and was ineffective for prevention of infection.[13] It should be noted that 62% ethanol, contained in many commercial hand sanitizers, is also ineffective for complete removal of rhinovirus from the hands and would be expected to be ineffective for the prevention of rhinovirus infection.

In vitro studies

Initial studies to screen a variety of organic acids approved for use in over-the-counter products revealed that salicylic acid, pyroglutamic acid and benzoic acid had potent virucidal activity that persisted for 3 h after application.[14] In these experiments, all of the acids were standardized to 0.145 M in 1:1 water/ethanol and the pH of each solution was adjusted to 3.0. The water/ethanol vehicle was used as the negative control. All three acids reduced the rhinovirus titre by at least 2 logs, to concentrations undetectable in the assay, 3 h after application to the skin. The log reduction in titre with each of these three acids was greater than that of 2% iodine at both the 1 h and 3 h time points.

In vivo studies

The efficacy of salicylic and pyroglutamic acid for prevention of experimental rhinovirus infection in human volunteers was examined in two randomized, double-blinded clinical studies.[14] Volunteers susceptible to rhinovirus type 39 applied a measured amount of a hand cleanser containing organic acid to both hands. The vehicle, 62% ethanol, was used as the negative control. At specified time points after application of the study material, the fingers of each hand were contaminated with 100 $TCID_{50}$ of rhinovirus type 39. After contamination with virus the hands were allowed to air dry for 10 min before the volunteers attempted to intentionally inoculate the virus on the right hand by making contact with both the conjunctiva and the nasal mucosa. Following the self-inoculation, the amount of virus contaminating the

Table 1. Effect of virucidal hand treatment on recovery of virus from the hands and infection of volunteers

Hand treatment	Interval between hand treatment and virus challenge	Recovery of virus from hands	Infection rate in volunteers
62% EtOH vehicle control (trial 1)	15 min	28/31 (90%)	10/31 (32%)
3.5% Salicylic acid	15 min	4/27 (15%)†	2/27 (7%)†
1% Salicylic + 3.5% pyroglutamic acid	15 min	0†	2/27 (7%)†
62% EtOH vehicle control (trial 2)	15 min	30/30 (100%)	9/30 (30%)
4% Pyroglutamic acid	15 min	5/30 (17%)*	4/30 (13%)
4% Pyroglutamic acid	1 h	8/30 (27%)*	4/30 (13%)
4% Pyroglutamic acid	3 h	19/32 (59%)*	7/32 (22%)

†$P < 0.05$ for comparison to vehicle control for trial 1.
*$P < 0.05$ for comparison to vehicle control for trial 2.

JAC antiviral

fingers of the left hand was determined by elution into 2 mL of viral collecting broth for quantitative culture. In the interval between the hand treatment and the virus challenge the volunteers remained at the study site and were not allowed to use or wash their hands.

The first study examined the effect of 3.5% salicylic acid, and a combination of 1% salicylic acid with 3.5% pyroglutamic acid at a single time point (15 min) after application. Both of the organic acids significantly reduced recovery of virus from the hands and the rhinovirus infection rate (Table 1). In the second clinical trial, a formulation of 4% pyroglutamic acid was tested by challenging with virus 15 min, 1 h and 3 h after application. In this experiment, recovery of virus from the hands was significantly reduced at all time points but the infection rate in the volunteers was not significantly different (Table 1).

Comment

The persistence of the virucidal effect of the organic acids for up to 3 h after application is an attractive feature of this approach. It is not clear, however, whether this persistence will be retained when studies are conducted under natural conditions. In these preliminary studies, volunteers were not permitted to use their hands in the interval between application of the acids and the challenge with virus. A limited evaluation found no effect of using the hands on virucidal activity in these studies,[14] but previous work suggests that the virucidal effect of the organic acids may be diminished by normal usage.[13]

In summary, there is substantial evidence that direct contact followed by self-inoculation of the respiratory mucosa is an important mechanism of transmission for the rhinoviruses. In light of this evidence, treatment of the hands with virucidal agents appears to be a rational approach to prevention of rhinovirus infection. These early studies suggest that the organic acids have potent virucidal activity against the rhinoviruses and may be suitable agents for this use. Ultimately, however, studies in the natural setting will be required to establish the effectiveness of this intervention.

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Exhibit K

Submitted as Exhibit to Amendment of January 28, 2009

Rhinovirus - Material Safety Data Sheets (MSDS)

MATERIAL SAFETY DATA SHEET - INFECTIOUS SUBSTANCES

SECTION I - INFECTIOUS AGENT

NAME: ***Rhinovirus***

SYNONYM OR CROSS REFERENCE: Acute viral rhinitis, acute coryza, common cold virus

CHARACTERISTICS: *Picornaviridae*; icosahedral, about 27 nm diameter, ss positive sense RNA, non-lipid enveloped capsid

SECTION II - HEALTH HAZARD

PATHOGENICITY: Most frequent cause of the common cold responsible for 30-50% of cases; acute infection of the upper respiratory tract; characterized by coryza, sneezing, lacrimation, irritated nasopharynx, headache, sore throat, chilliness and malaise lasting 2-7 days; little or no fever; can be accompanied by laryngitis, tracheitis and bronchitis; secondary bacterial infection may produce acute otis media, sinusitis or pneumonitis

EPIDEMIOLOGY: Worldwide; occurs mostly in the early fall and winter; over 100 recognized serotypes; incidence highest in children < 5 years

HOST RANGE: Humans

INFECTIOUS DOSE: Ranges from 0.032 to 0.4 $TCID_{50}$ when given as nasal spray

MODE OF TRANSMISSION: Direct contact or inhalation of infectious airborne droplets; indirect spread by contaminated hands and freshly soiled articles

INCUBATION PERIOD: From 2-4 days

COMMUNICABILITY: Nasal discharges taken 24 hours before and 5 days after onset have been shown to be infectious

SECTION III - DISSEMINATION

RESERVOIR: Humans

ZOONOSIS: None

VECTORS: None

SECTION IV - VIABILITY

DRUG SUSCEPTIBILITY: No specific antivirals although sensitive to alpha-2 interferon

SUSCEPTIBILITY TO DISINFECTANTS: Susceptible to l.0% sodium hypochlorite, tincture of iodine, phenol-alcohol disinfectant, 2% glutaraldehyde; resistant to organic solvents, ethanol

PHYSICAL INACTIVATION: Inactivates at pH < 3.6

SURVIVAL OUTSIDE HOST: Relatively stable at 24-35° C; survives for days on environmental surfaces; survives on the human skin up to 3 hours; survival in aerosols enhanced by low temperature and high relative humidity

SECTION V - MEDICAL

SURVEILLANCE: Identification by tissue culture

FIRST AID/TREATMENT: Rest, hydration, nasal decongestant and saline gargles

IMMUNIZATION: None

PROPHYLAXIS: Antiviral agents have proven effective in vitro, but with little effect in clinical trials

SECTION VI - LABORATORY HAZARDS

LABORATORY-ACQUIRED INFECTIONS: None reported although probably quite common

SOURCES/SPECIMENS: Nose and throat secretions

PRIMARY HAZARDS: Droplet exposure of mucous membranes; indirect via hands

SPECIAL HAZARDS: None

SECTION VII - RECOMMENDED PRECAUTIONS

CONTAINMENT REQUIREMENTS: Biosafety level 2 practices and containment for activities utilizing infectious body fluids, tissues and cultures

PROTECTIVE CLOTHING: Laboratory coat; gloves when skin contact with infectious materials is unavoidable

OTHER PRECAUTIONS: None

SECTION VIII - HANDLING INFORMATION

SPILLS: Allow aerosols to settle; wearing protective clothing gently cover spill with absorbent paper towel and apply 1% sodium hypochlorite starting at perimeter and working towards the center; allow sufficient contact time (30 min) before clean up

DISPOSAL: Decontaminate all wastes before disposal; steam sterilization, chemical disinfection, incineration

STORAGE: In sealed containers that are appropriately labelled

SECTION IX - MISCELLANEOUS INFORMATION

Date prepared: February, 2001

Prepared by: Office of Laboratory Security, PHAC

Although the information, opinions and recommendations contained in this Material Safety Data Sheet are compiled from sources believed to be reliable, we accept no responsibility for the accuracy, sufficiency, or reliability or for any loss or injury resulting from the use of the information. Newly discovered hazards are frequent and this information may not be completely up to date.

Date Modified: 2001-09-27